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## GLYOXALASE

### III. GLYOXALASE AS A REAGENT FOR THE QUANTITATIVE MICRO-ESTIMATION OF GLUTATHIONE

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(Received for publication, December 10, 1934)

Reduced glutathione was first shown by Lohmann (1) to be a specific activator of the enzyme glyoxalase, which converts methylglyoxal into lactic acid. The amount of activation was dependent, within a certain range, upon the concentration of glutathione. On the basis of this relationship a new manometric method for the estimation of glutathione has been developed. The amount of glutathione may be determined from the amount of activation it produces with a given quantity of enzyme.

It was apparent from the work of Lohmann on muscle and liver glyoxalase, and of Platt and Schroeder (2) on acetone-yeast glyoxalase, that greater changes in activity were produced by increasing amounts of glutathione where low concentrations of the latter were used. At higher concentrations the activity approached a maximum beyond which further increases in glutathione gave no increased activity. Therefore, to use the activity produced as a measure of the glutathione present, it is essential to work in the range of low concentrations.

The extreme specificity of glutathione for this reaction was pointed out by Lohmann. Only the reduced form has any effect. Oxidized glutathione, cysteine, thioglycolic acid, hydrogen sulfide, hydrogen cyanide, pyrophosphate, citrate, and hydroxyquinoline had no activating effect whatsoever. To this list thioneine and ascorbic acid, two substances which are known to react in iodometric methods, are now added. Thus this specificity provides us with a means of distinguishing glutathione, particularly in tissues, from other sulfhydryl compounds and ascorbic acid.

## EXPERIMENTAL

Glyoxalase activity measurements are made in the simple Barcroft-Warburg manometric apparatus at 25°, the manometer cups having a single side bulb of about 0.8 cc. capacity and a total volume of about 18 cc. The manipulation of the apparatus as regards glyoxalase activity measurement has been discussed at length in Paper I of this series (2) and in general the same procedure is followed in this work. Since the factor being studied here is glutathione, this is always placed in the side bulb. In setting up a run, materials are measured first into the main chamber of each manometer cup as follows: 0.5 cc. of glutathione-free acetone-yeast (15 to 20 per cent suspension), 0.2 cc. of methylglyoxal (10 mg. per cc.), 0.4 cc. of sodium bicarbonate (0.2 M), and water to make a total volume of 2 cc. including the measurements in the side bulb. By mixing the yeast and methylglyoxal first, the observed blank gas evolution caused by a slight reaction between these two is cut down, since the greater part of this reaction occurs before the manometric readings can be started.

The acetone-yeast to be used as the source of glyoxalase must be glutathione-free. Such a product may be prepared by simply washing the usual acetone-yeast as prepared by the method of Albert, Buchner, and Rapp (3). A small amount may be washed free of glutathione each time as described in the previous paper (2), or a large amount may be washed and subsequently dried by the acetone-ether procedure. In this form it is more convenient for use, as much time is saved at the start of each day's work. For use in the manometric estimations an amount of yeast is chosen which, with 0.1 mg. of glutathione, will give in 20 minutes an amount of CO<sub>2</sub> equal to about three-quarters of the capacity of the manometers (in our case about 200 to 250 c.mm.). With our yeast preparations 0.5 cc. of a 15 or 20 per cent suspension usually contained the desired amount of enzyme. Exact duplication of the yeast measurement is very essential; therefore, thorough mixing and accuracy of measurement are important.

In order to obtain methylglyoxal solutions of fairly high concentration the procedure of Bernhauer and Görlich (4) was used. The methylglyoxal content was determined as the *m*-nitroben-

zoylosazone (5) and the solution diluted so as to contain 10 mg. per cc.

*Effect of Protein Precipitating Agents on Acetone-Yeast Glyoxalase*—Since it is usually desired to analyze for glutathione in a protein-free filtrate, and since sulfosalicylic and trichloroacetic acids, because of their acidity, seem to be the best reagents for preparing such filtrates, the effect of these acids as regards their toxicity toward glyoxalase was first investigated. A definite amount of pure glutathione in the acid was measured into the side bulb of the manometer cups along with a control containing the same amount of glutathione but no acid. An amount of 0.2 M bicarbonate was added to the side bulb containing the acid in an amount sufficient to neutralize it to methyl orange, this amount being previously determined by a separate titration. Then, after saturating the mixtures in the manometers with 5 per cent  $\text{CO}_2$  in nitrogen and tipping the contents of the side bulb into the yeast, methylglyoxal, and bicarbonate mixture in the main part of the cup, the effect on the enzyme could be determined. It was found that amounts of neutralized sulfosalicylic acid up to 0.5 cc. of 2 per cent acid had no effect on the enzyme. 0.2 cc. of trichloroacetic acid, however, showed a slight toxic effect, this effect being greater with increasing quantities of the acid. Sulfosalicylic acid filtrates were therefore used in determining the glutathione content of blood and tissues.

*Effect of Bicarbonate Concentration*—Since there might be slight variations in the neutralization of the acid in the side bulb, the effect of an excess bicarbonate concentration was investigated. It was found that there was no difference in the amount of activation observed when the total quantity of bicarbonate present was varied over the range of 0.3 to 0.6 cc. The activity was somewhat less when only 0.2 cc. was used. The optimum pH range was therefore quite wide, 7.5 to 7.8, and slight variations in the amount of bicarbonate used to neutralize the acid in the side bulb could thus have no effect on the activity. It is better, however, to have the acid slightly overneutralized, for the unneutralized sulfosalicylic acid is sometimes slightly toxic.

*Specificity of Glutathione*—Since thioneine, ascorbic acid, and cysteine are thought to be the chief substances present in tissues which interfere in the usual methods of glutathione estimation,

their effect on the glyoxalase method was studied. No activating effect whatsoever was observed, either in the presence or absence of glutathione.

A further indication of the specificity of glutathione for glyoxalase activation is given in the succeeding paper (6) in which it is shown that the glutathione molecule may be attacked in such a way by antiglyoxalase that it becomes inactive towards glyoxalase, yet the sulfhydryl group remains intact. Lohmann reported a large number of other substances which had no activating effect on the enzyme.

*Analytical Procedure Adopted*—The method in brief is as follows: A standard glutathione curve is first established by determining the effect of increasing known amounts of pure glutathione on the activity of glutathione-free acetone-yeast glyoxalase. A portion of the solution to be analyzed is then added to a similar amount of yeast and the activating effect observed. By reference to the standard curve the quantity of glutathione present can be determined.

The glutathione standards run usually contain 0.025, 0.05, 0.1, and 0.15 mg. of glutathione in solution in the side bulb. A yeast blank with no glutathione is also included in the series and its value subtracted from all the other readings. This blank takes care of any pressure developed because of side reactions between methylglyoxal and yeast, as well as of changes in pressure due to variations in temperature or barometric pressure. After tipping, an open period of 4 minutes is used. Readings are then taken every 5 minutes for 20 minutes. If the  $\text{CO}_2$  produced in each standard (after subtraction of the blank) is plotted against time, a straight line cutting the origin should be obtained; in other words, the amount of  $\text{CO}_2$  produced every 5 minutes should remain constant. If such is not the case, but the activity remains linear for the first 15 minutes, the correct 20 minute figure may be determined by extrapolation. The amount of  $\text{CO}_2$  produced in 20 minutes is then plotted against the amount of glutathione and the standard glutathione curve traced through these points. Fig. 1 is an example of such a curve.

For the estimation of an unknown amount of glutathione, an amount of solution containing not over 0.1 mg. is placed in the side bulb and the activity it produces with the same amount of

yeast is measured in the same manner as with the standards. If only one or two samples are to be analyzed these may be run in the same series as the standards, the 0.15 mg. standard being omitted when the set-up contains only six manometers. If the analyses are run as a separate series, a yeast blank must be repeated here.

When a sulfosalicylic acid protein-free filtrate is used for the analysis, this must be neutralized in the side bulb by adding sufficient 0.2 M bicarbonate to neutralize to methyl orange, this

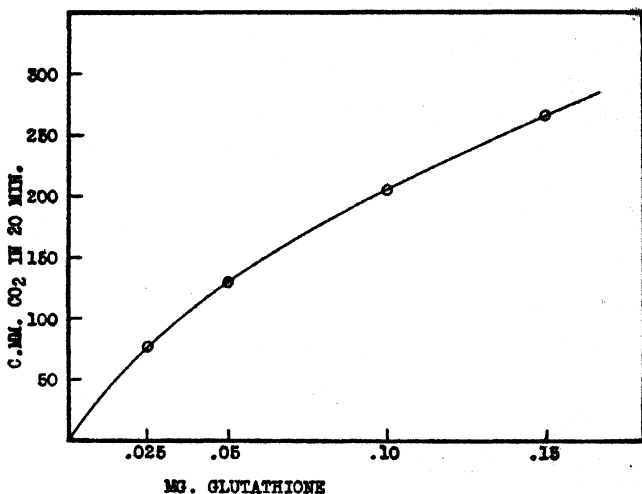


FIG. 1. Standard glutathione curve

amount having been determined by a separate titration. We have found convenient the following amounts of 2 per cent sulfosalicylic acid blood and tissue filtrates.

Whole blood.....	0.5	cc. 1:5	filtrate
Red blood cells.....	0.5	" 1:10	"
Blood plasma.....	0.5	" 1:1.5	"
Animal tissues.....	0.25	" 1:5	"

After measuring the amount of CO<sub>2</sub> produced in 20 minutes by the unknown, the standard curve is referred to and the amount of glutathione present read off directly.

*Recovery Experiments*—Recovery experiments for glutathione added to liver extract, blood plasma, red blood cells, whole blood, and yeast have been satisfactory. Table I presents such results.

*Comparison of Manometric and Iodometric Values for Glutathione*—A few analytical results obtained by the new method are given in Table II together with the glutathione values obtained by iodometric titration. For the titration the iodate procedure of Woodward and Fry (7) was used: in the case of blood, on 1:5 or 1:10 dilution in 2 per cent sulfosalicylic acid; in the case of

TABLE I  
*Recovery of Glutathione Added to Blood and Tissues*

Material to which GSH was added	Original GSH content of amount used	GSH added per manometer cup	Total GSH estimated	GSH recovered	Recovery
	mg.	mg.	mg.	mg.	per cent
Rabbit liver extract.....	0	0.1	0.099	0.099	99
“ “ “ .....	0	0.1	0.098	0.098	98
“ “ “ .....	0	0.05	0.050	0.050	100
Blood plasma, human, cancer.....	0	0.067	0.067	0.067	100
“ “ rabbit.....	0	0.067	0.068	0.068	101
Red blood cells, human, cancer....	0.032	0.05	0.080	0.048	96
“ “ “ “ “ .....	0.099	0.05	0.146	0.047	94
“ “ “ “ normal....	0.016	0.03	0.0455	0.0295	98
“ “ “ “ “ .....	0.016	0.02	0.035	0.019	95
Whole blood, human, normal.....	0.0305	0.05	0.081	0.0505	101
“ “ “ “ “ .....	0.028	0.05	0.078	0.050	100
“ “ “ “ “ .....	0.036	0.05	0.087	0.051	102
Acetone-yeast.....	0	0.05	0.049	0.049	98

tissues, on a filtrate similar to the Okuda and Ogawa (8) sulfosalicylic acid filtrate. While this titration method was not recommended for determining the glutathione of tissues, nevertheless it may be used for the purpose of determining the total iodine-consuming power of the extracts. A comparison of such a value with a true glutathione value is of interest in view of the fact that iodometric methods have been most extensively used for glutathione measurements in tissues.

Ascorbic acid values obtained by 2,6-dichlorophenol indophenol titration have also been included in Table II, since this substance

is a constituent of tissues which reacts with iodine and is therefore included in any iodometric figure for glutathione. For this estimation, the titration procedure of Birch, Harris, and Ray (9) was used on the sulfosalicylic acid filtrates as described above. Sulfosalicylic acid seems superior to trichloroacetic acid for such a titration as the end-point is sharper, there is less fading of the indicator, and the autoxidation of ascorbic acid is considerably cut down.

From Table II it may be observed that the iodate value is, on the whole, considerably larger than the manometric value; in the case of adrenal tissue the iodate value is about 10 times the true value. In agreement with the work of Boyland (10) is the finding that tumors contain only about one-third as much glutathione as indicated by iodometric titration. With blood, however, there is quite close agreement, indicating that with the iodate titration procedure for blood only glutathione is estimated.

If glutathione and ascorbic acid-like material are the only substances present which react with iodine in the iodate titration for glutathione, the sum of these two (Column 5) should be the same as the iodate titration value (Column 1). There is fair agreement in many cases, as was also found by Boyland. The lack of agreement in certain cases may probably be accounted for by the uncertainty of the iodine end-point in the tissue extracts.

*Oxidized Glutathione*—It was hoped that it might be possible to show by the new manometric method whether the increase in reducing material caused by zinc reduction of an acid extract of tissue or blood was due to reduced glutathione. When, however, a zinc-treated sulfosalicylic acid filtrate was used, no activity whatsoever was obtained, due to the high toxicity of the zinc to the enzyme. Lohmann had previously reported zinc to be non-toxic to glyoxalase, but his highest concentration was 0.002 M, while in the zinc-treated sulfosalicylic acid the concentration is considerably higher. Several attempts to remove the zinc, and yet allow a quantitative recovery of added glutathione, failed.

The reduction of oxidized glutathione by other metals in the presence of acid was therefore investigated, together with toxicity measurements of the metal toward the enzyme. Magnesium had previously been used, as well as zinc, as a means of reducing oxidized glutathione in an acid medium. When magnesium-treated



sulfosalicylic acid was found to be entirely non-toxic to glyoxalase, considerable hope was offered for the estimation of oxidized

TABLE II

*Comparison of Manometric and Iodate Values for Glutathione in Blood and Tissues*

Tissue	GSH value (iodate)	GSH (manometric)	Ascorbic acid value (2,6-dichlorophenol indophenol)	GSH equivalent of as- corbic acid	GSH equivalent of GSH + ascorbic acid	Per cent of iodate GSH value due to GSH + ascorbic acid
	(1)	(2)	(3)	(4)	(5)	(6)
	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	
Liver, rat, normal.....	307	236	25	87	323	105
" " ".....	261	180	19	66	246	94
" " ".....	233	116				
" " Philadelphia No. 1 sarcoma.....	169	122	10.5	37	169	100
" " " " 1 ".....	189	132				
" " " " 1 ".....		176	14			
Tumor, rat, " " 1 ".....	215	86	53	185	271	126
" " " " 1 ".....		72	47			
" " " " 1 ".....	154	68				
" horse, melanosarcoma.....	145	47	26	91	138	95
" " ".....	77	34	13	45	79	103
Muscle, " ".....	66	42	<1	0	42	64
Adrenal, " ".....	960	94	243	850	944	98
Brain, rat, Philadelphia No. 1 sarcoma.....	164	45	28.5	99.4	144	88
" " " " 1 ".....		42	35			
Spleen, rat, " " 1 ".....		100	34			
" " normal.....	197	100	38	133	233	118
Kidney, rat, Philadelphia No. 1 sarcoma.....		98	13			
" " normal.....	253	134	15	52	186	73
Red blood cells, human, normal.....	75	76	0	0	76	101
" " " " ".....	72	68	0	0	68	94
" " " " cancer.....	61	56	0	0	56	92
" " " " ".....	81	80	0	0	80	99
" " " horse, melanosarcoma.....	94	100	0	0	100	106

glutathione. However, when pure oxidized glutathione was used, it was not possible to bring about reduction of more than a small

fraction in any concentration of sulfosalicylic, sulfuric, or trichloroacetic acids. This was very surprising in view of the fact that both Schelling (11) and Thompson and Voegtlin (12) found an increase in the reducing value of tungstic and trichloroacetic acid filtrates of blood and tissues after magnesium treatment.

Other metals investigated were cadmium, manganese, calcium, and sodium. Manganese was considerably more effective than magnesium in reducing oxidized glutathione, although less powerful than zinc. However, it was found to be slightly toxic to glyoxalase and was therefore abandoned. Cadmium also was not suitable as its reaction with sulfosalicylic acid to give hydrogen was too slow. For this reason metals below cadmium in the electromotive series were not tried. Going up the electromotive series, we found calcium and sodium (amalgam) were even less effective than magnesium for reduction of the oxidized glutathione. Although there is great evolution of hydrogen, the reduction does not seem to depend upon this but upon the potential of the metal, for the degree of reduction by the metal is in the order of the electromotive series.

From these considerations, therefore, zinc seems to be the only metal suitable for reduction of oxidized glutathione in acid extracts of tissue, and due to its toxicity toward glyoxalase we have not been able to apply it to the manometric method.

#### DISCUSSION

The method described here should prove of considerable value when the true glutathione content of the tissues is desired, as no other substance so far as is known can be confused with glutathione in this method. As small an amount of glutathione as 0.01 mg. in 0.5 cc. of solution may be satisfactorily determined. The accuracy of estimation is extremely good, the limit of error probably not being over 6 per cent. It depends to a large extent upon the accuracy of measurement of the yeast and of the glutathione in determining the standard curve.

Owing to the complexity of the apparatus and technique this method can hardly be recommended for routine examination. The iodate method for blood is still to be preferred and its accuracy has been shown by comparison with the above method.

## SUMMARY

A manometric method for reduced glutathione in tissues has been developed. It depends upon the measurement of the activating effect of glutathione on acetone-yeast glyoxalase.

The effect of glutathione is specific, neither cysteine, thioneine, ascorbic acid, nor oxidized glutathione (among other substances) producing any activity.

Glutathione values in tissues by this method are considerably lower than by iodometric titration. In blood there is not this discrepancy.

2,6-Dichlorophenol indophenol titrations in most cases account for the difference between the iodometric and manometric values for glutathione.

The author wishes to acknowledge her indebtedness to Dr. E. F. Schroeder for his advice throughout the course of this investigation.

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## GLYOXALASE

### IV. THE ANTIGLYOXALASE ACTION OF KIDNEY AND PANCREAS PREPARATIONS

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In a previous paper of this series (1) evidence was presented for the occurrence, in rat kidney, of a powerful inhibitor of the enzyme glyoxalase. The behavior of this inhibitor is similar in some respects to that of antiglyoxalase, found by Dakin and Dudley (2) to occur in pancreas. The results described in the present paper confirm our previous observations and throw light on the mechanism of the inhibition.

Although numerous investigations have been carried out, comparatively little is known of the nature of pancreatic antiglyoxalase. Foster (3) and von Vargha (4) doubted the existence of a specific inhibiting factor, and suggested that the observed effects were due merely to destruction of substrate by proteins and other amino compounds present in the pancreas preparations. Ariyama (5) and Ariyama and Kobayashi (6), however, showed that the losses in substrate due to the presence of amino compounds were far too small to account for the entire inhibiting effect. Dakin and Dudley (2) found an antiglyoxalase effect in aqueous extracts, as well as in dry preparations, from a wide variety of animals, the inhibition being non-specific for the species. The inhibitor was found to be thermolabile, non-dialyzable through animal membranes, fairly stable towards 1 per cent sodium carbonate, but destroyed by 0.01 N HCl. The effect was not due to trypsin, lipase, or amylase. When enzyme and pancreatin solutions were allowed to stand together before addition of substrate, the degree of inactivation of the glyoxalase increased with the

time of preliminary incubation. From this it was concluded that the effect of the inhibitor was exerted on the enzyme rather than on the substrate, and that the reaction responsible for the inhibition proceeded rather slowly. Ariyama (5) and Sakuma (7) reported a considerably greater thermostability for the inhibitor; pancreatin, for example, did not lose its effect even when heated to 120° for 30 minutes. The enzymic nature of the inhibitor thus seemed doubtful. Kuhn and Heckscher (8) were able to adsorb pancreatic antiglyoxalase from a glycerol extract of pig pancreas with alumina C<sub>γ</sub> at pH 3.8, but no means of elution was found. Girsavicius (9) has recently obtained evidence that the antiglyoxalase of pancreatin exerts its effect by destroying the coenzyme glutathione. Since no loss of titratable sulfhydryl occurred, he suggested that the effect might be due to hydrolytic splitting of the glutathione molecule. He also found that the inhibition began only after the reaction mixtures had been subjected to a preliminary incubation period of about 2 hours, and then increased rapidly. It should be pointed out that pancreatic antiglyoxalase is quite distinct from the pancreatic factor which inhibits glycolysis. This latter factor was discovered by Winfield and Hopkins (10) and later shown by Case and McCullagh (11) to be identical with amylase.

By means of the manometric method previously described (1, 12) we have been able to demonstrate the presence of an antiglyoxalase in kidney, as well as pancreas, of rat, rabbit, pig, and horse. Acetone-ether-dried preparations of these organs also contain the inhibitor. The effect is non-specific for the species, both kidney and pancreas preparations from the above animals inhibiting glyoxalase obtained from liver, muscle, or acetone-yeast. The full effect of the inhibitor is not exerted instantaneously. In each case the degree of inhibition of glyoxalase activity increases with time. The more inhibitor present, the more rapid is the inactivation.

The inhibitor appears to be inactive in intact slices of kidney and pancreas tissue, exerting its effect only after the cell structure has been destroyed in the process of extraction. In a previous paper (1) it was shown that intact slices of these tissues have a high glyoxalase activity, that of kidney even exceeding that of liver slices. The rate of the enzyme reaction shows no appreciable

falling off for at least 1 hour. But when aqueous extracts of kidney and pancreas are used, the glyoxalase either is inactive, or decreases very rapidly in activity. Extracts of liver and spleen, on the other hand, have a high glyoxalase activity which remains constant for long periods of time. These findings indicate that the action of antiglyoxalase is in some manner related to the tissue structure.

Conclusive evidence has been obtained that kidney antiglyoxalase destroys the coenzyme glutathione, and does not affect the enzyme itself. Also, the effect is not on the sulfhydryl group, but on some other part of the glutathione molecule. Pure glutathione, when incubated with kidney preparations, loses its ability to activate glyoxalase although no loss of titratable sulfhydryl occurs. These findings agree with the suggestion made by Girsavicius on the antiglyoxalase action of pancreatin. However, a closer examination of the behavior of pancreatin and fresh pancreas extracts has shown that the amount of inhibition produced is considerably greater than can be accounted for by the loss of glutathione. Our results indicate that pancreas may contain two antiglyoxalase factors, one of which resembles that of kidney in acting on the glutathione (but not on the sulfhydryl group) and a second which probably acts on the enzyme itself.

A method has been devised for the quantitative determination of antiglyoxalase activity. Preliminary results indicate that kidney cortex contains about twice as much inhibitor per unit weight as does the medulla. Kidney extracts generally produce a much greater inhibition than do corresponding amounts of pancreas extracts. Fresh rat kidney, the most active source thus far found, contains more than 20 times as much antiglyoxalase as does pancreatin. Further work is in progress on the distribution and properties of both kidney and pancreas antiglyoxalases.

#### EXPERIMENTAL

##### *Inhibition of Glyoxalase Activity by Kidney and Pancreas Preparations*

For demonstrating the inhibiting effect of kidney and pancreas preparations on glyoxalase activity, the manometric method previously described (1, 12) was used throughout. Kidney, pancreas, liver, and muscle extracts were prepared by grinding the

tissues with sand, and extracting for 30 minutes at room temperature with 5 parts of water. The mixtures were then centrifuged. Glyoxalase activity determinations were made at 25°, with 2 mg of methylglyoxal and 0.4 cc. of 0.2 M sodium bicarbonate, in a total volume of 2 cc. Blank determinations were made on the glutathione-free enzyme and inhibitor solutions, and the values deducted when significant.

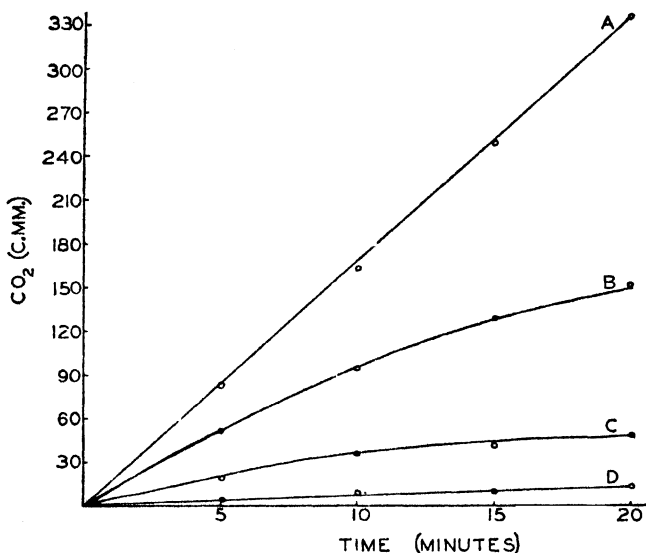


FIG. 1. Effect of increasing amounts of rat kidney extract on the activity of rabbit liver glyoxalase. Curve A, liver control; Curve B, 0.1 cc. of kidney extract; Curve C, 0.2 cc. of kidney extract; Curve D, 0.4 cc. of kidney extract.

Fig. 1 shows the effect of increasing amounts of rat kidney extract on the activity of rabbit liver glyoxalase. The indicated amounts of 1:5 kidney extract were added to 0.4 cc. of fresh, active 1:5 liver extract, and the glyoxalase activity determined as rapidly as possible (without added glutathione). The time of contact of the kidney extract with the liver glyoxalase before the start of the readings did not exceed 10 minutes. Nevertheless, complete inhibition of the enzyme activity had occurred in the

mixture containing 0.4 cc. of kidney extract, and partial inhibition in the others. Similar mixtures incubated for 40 minutes at 25° were found to be completely inactivated, even with 0.1 cc. of kidney extract.

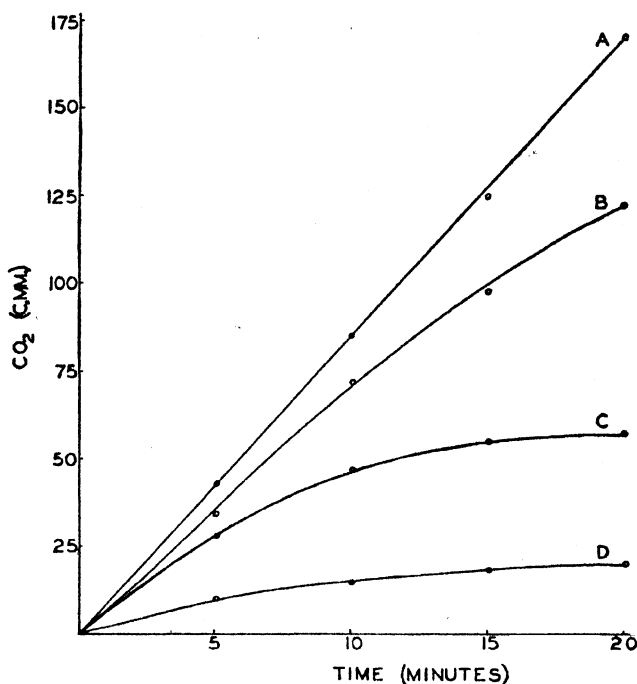


FIG. 2. Inhibition of acetone-yeast glyoxalase activity by rabbit kidney and pancreas extracts; effect of time. Curve A, acetone-yeast control; Curve B, 0.4 cc. of pancreas extract (immediate); Curve C, 0.4 cc. of kidney extract (immediate); Curve D, same as for Curve B, but 3 hours incubation at 37°.

Fig. 2 shows the inhibiting effect of rabbit kidney and pancreas extracts on unwashed (glutathione-containing) acetone-yeast, prepared as previously described (12). The indicated amounts of 1:5 kidney and pancreas extracts were added to 0.4 cc. of a 10 per cent suspension of acetone-yeast, and the glyoxalase activity determined immediately. In the case of pancreas, a similar reac-



tion mixture was incubated for 3 hours at 37°, and the glyoxalase activity then determined. Kidney and pancreas extracts behave similarly in that the inhibition produced increases with time, and with the concentration of inhibitor. No evidence was found for the existence of an induction period before inhibition sets in, as claimed by Girsavicius for antiglyoxalase from pancreatin. The effect can be observed immediately provided enough inhibitor is present. Similar results were obtained from fresh pancreas and kidney extracts of horse and pig, and with pancreatin (Parke Davis, Mulford) and dried pig kidney prepared by mincing and repeated treatment with acetone and ether. Muscle glyoxalase is also inhibited by these various preparations.

#### *Determination of Antiglyoxalase Activity*

The following procedure has been adopted for obtaining an estimate of the quantity of inhibitor present in a given preparation. A yeast control is set up by determining the glyoxalase activity (c.mm. of CO<sub>2</sub> formed in 20 minutes at 25°) of a reaction mixture containing 0.5 cc. of a 20 per cent suspension of washed acetone-yeast, 0.4 cc. of 0.2 M sodium bicarbonate, 2 mg. of methylglyoxal, and 0.1 mg. of glutathione (side arm), in a total volume of 2 cc. After temperature equilibration and saturation of the reaction mixture with 5 per cent CO<sub>2</sub> in nitrogen, the contents of the side arm are tipped into the main chamber of the reaction vessel. The manometer stop-cock is now opened for exactly 2 minutes, then closed again, and the amount of CO<sub>2</sub> formed in 20 minutes is determined. Simultaneously a yeast blank is determined in the same manner, but in the absence of glutathione. The difference between the values obtained represents the yeast control (100 per cent activity).

For determining inhibiting power, a known volume of the inhibitor solution is added to a similar reaction mixture (glutathione in side arm) and the glyoxalase activity determined as before. At the same time an inhibitor blank is determined in the absence of acetone-yeast. The observed activity (c.mm. of CO<sub>2</sub> in 20 minutes) minus the yeast and inhibitor blanks represents the true glyoxalase activity of the mixture containing the inhibitor. The results are expressed as per cent of the control glyoxalase activity. It has been found that a linear relation exists between the log-

arithm of the observed glyoxalase activity (expressed as per cent of the control activity) and the quantity of inhibitor present. Fig. 3 shows this relation in the case of pancreatin and rabbit kidney. Due probably to inaccuracies involved in the method of measurement, the linearity does not always hold exactly when the

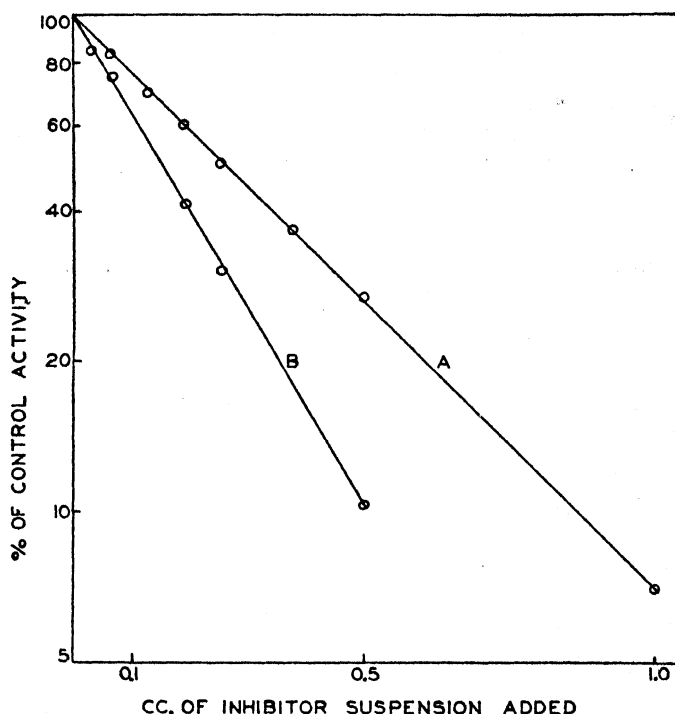


FIG. 3. Relation between glyoxalase activity and amount of antiglyoxalase present. Curve A, pancreatin (10 per cent suspension); Curve B, rabbit kidney (2 per cent suspension, acetone-ether-dried).

degree of inhibition is either very large or very small. Analytically, the relation is expressed by the equation  $\log A = 2 - cM$  where  $A$  is the observed glyoxalase activity (expressed as per cent of the control activity),  $M$  is the amount of inhibitor preparation added, and  $c$  is an empirical constant. For comparative purposes,

we may express the inhibiting power of various preparations in terms of the amount necessary to produce a definite reduction in glyoxalase activity. On this basis we define unit quantity of antiglyoxalase as that amount which results in a 50 per cent reduction in activity under the prescribed conditions. Experimentally, an amount of inhibitor preparation is used to produce a glyoxalase activity between 30 and 70 per cent of that of the control, and  $M_{50}$ , the amount containing 1 unit of antiglyoxalase, is calculated by means of the equation  $M_{50} = 0.3 M / (2 - \log A)$  where  $M$  and  $A$  are the experimental quantities.

No extended study has as yet been made of the quantitative occurrence of antiglyoxalase in kidney and pancreas of various

TABLE I  
*Antiglyoxalase Activity of Kidney and Pancreas Preparations*

Tissue	Antiglyoxalase per gm. tissue (dry weight)
	units
Pancreatin.....	40
Pig kidney (acetone-ether-dried).	136
pancreas (fresh extract).	56
kidney     "     "	160
Horse pancreas (fresh extract).	19
kidney medulla (fresh extract).	76
cortex     "     "	186
Rabbit kidney (fresh extract).	304
Rat kidney (fresh extract).....	980

animals. Some of our results to date are presented in Table I. The values are expressed as units of antiglyoxalase per gm. of tissue extracted (dry weight, calculated as 20 per cent of the wet weight). In the case of pancreatin and acetone-ether-dried kidney, aqueous suspensions were used. Acetone-ether treatment apparently diminishes the inhibiting power.

#### *Mechanism of Antiglyoxalase Action*

*Kidney*—Although no estimations of substrate losses were made, it is quite improbable that reaction between methylglyoxal and amino groups plays any significant part in the inhibition. For example, the kidney extract used in the experiment illustrated in

Fig. 1 gave a blank  $\text{CO}_2$  evolution of only 8 c.mm. in 20 minutes, with methylglyoxal and bicarbonate (0.4 cc. of 1:5 kidney extract). Since any reaction of methylglyoxal with amino groups would lead to an increase in acidity, and therefore to  $\text{CO}_2$  evolution, it is obvious that such a reaction has not occurred to any appreciable extent. Blanks obtained with pancreas extracts are slightly higher, but still not enough to account for any significant loss of methylglyoxal.

A number of experiments were carried out to determine whether the kidney inhibitor exerts its effect on the enzyme itself, or on the coenzyme glutathione. Varying amounts of 1:5 rabbit kidney extract were added to constant amounts of active rabbit liver extract, the mixtures being made up to the same final volume with water and allowed to stand at  $5^\circ$ . This temperature was chosen to minimize losses of glyoxalase activity which occur when the enzyme stands for long periods at higher temperatures. At intervals, portions were removed (corresponding to 0.4 cc. of liver extract) and the glyoxalase activity determined without added glutathione. Simultaneously, glutathione analyses were made on separate portions of the reaction mixtures after removal of protein with an equal volume of 4 per cent sulfosalicylic acid. It was found that glutathione losses occurring during the incubation of the reaction mixtures could not be accurately followed by the usual iodometric methods because of the formation of considerable amounts of other iodine-consuming substances. A manometric method, described in detail in the preceding paper (13), was therefore used. The method depends upon the measurement of the specific activating effect of glutathione on a constant amount of acetone-yeast glyoxalase.

The results are given in Table II in terms of per cent loss of glyoxalase activity and of glutathione with time, as compared to the liver control. This liver control value represents the glyoxalase activity and glutathione content of 0.4 cc. of liver extract (no kidney present), as determined at the time the incubation mixtures of liver plus kidney were set up. As will be seen from Table II, the glyoxalase activity of liver extract, incubated at  $5^\circ$ , decreases considerably with time, but not to the same extent as that of the liver plus kidney mixtures. For low concentrations of glutathione, such as were present in the liver extract used, the

glyoxalase activation is very nearly directly proportional to the glutathione concentration. If the inhibitor acts only on the glutathione, the per cent loss of the latter should agree closely with the per cent activity loss. Inspection of Table II shows that such agreement does in fact exist. At first glance it would appear that this agreement might be due to equal inhibiting effects of a non-protein kidney extractive on the glyoxalases of liver and acetone-yeast, the glutathione determination depending, as it does, on the degree of activation of acetone-yeast glyoxalase produced by the deproteinized reaction mixtures. This possibility is, however,

TABLE II

*Relation between Loss of Glutathione and Inhibition of Liver Glyoxalase Activity by Kidney Extract*

Kidney extract per 100 cc. liver extract	Time of incubation	Loss of glyoxalase activity	Loss of glutathione
cc.	hrs.	per cent	per cent
0 (Liver control)	2.5	17.4	19.1
	4.5	38.6	38.2
1.56	0	24.2	
	2.5	59.2	50.0
	4.5	79.7	78.0
0.78	0	13.1	
	2.5	35.8	33.8
	4.5	56.2	57.4
0.39	0	6.0	
	2.5	26.3	32.3
	4.5	47.8	53.0

ruled out by the fact that the sulfosalicylic acid treatment completely removes the antiglyoxalase from solution, while all the glutathione passes into the filtrate. As previously pointed out, neither can losses in substrate account for the inhibiting effects observed. It therefore appears probable that the antiglyoxalase exerts its effect only on the glutathione. This assumption is supported by the following experiments.

Table III shows that kidney antiglyoxalase has no effect on the enzyme itself. Glutathione-free liver extract (prepared by oxygenation of fresh extract) was allowed to stand at 5° with varying amounts of 1:5 rabbit kidney extract. At intervals portions corresponding to 0.4 cc. of liver extract were removed and the

glyoxalase activity determined in the presence of 0.25 mg. of added glutathione. The results are expressed as per cent loss of glyoxalase activity compared to the liver control without inhibitor. As shown by the values for 0 time of incubation, considerable inhibition occurs during the 20 minutes required to make the determination. Further incubation, however, does not increase the amount of inhibition obtained. This indicates clearly that the effect of the inhibitor is not on the enzyme.

When glutathione-containing liver extract or acetone-yeast is incubated with kidney extract until the glyoxalase becomes inactive, the latter may be reactivated by addition of pure glutathione. As is the case in the experiment recorded in Table III, the original glyoxalase activity is not completely restored by the

TABLE III  
*Effect of Kidney Antiglyoxalase on Glutathione-Free Glyoxalase*

Time of incubation	Loss of glyoxalase activity		
	Sample A	Sample B	Sample C
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0	63.4	42.0	18.5
4	63.9	41.5	16.0
22	61.0	37.3	18.3

Samples A, B, and C contained respectively 1.0, 0.5, and 0.25 cc. of 1:5 kidney extract per cc. of liver extract.

addition of the quantity of glutathione originally present. This is to be expected, since the antiglyoxalase still present will at once begin to react with the glutathione added, thus decreasing the activating effect of the latter.

Table IV shows that the per cent inhibition of glyoxalase activity by kidney extract is independent of the quantity of enzyme present, provided that constant amounts of glutathione and inhibitor are used. Such a result is to be expected if the inhibitor acts on the glutathione alone. Increasing amounts of a 10 per cent suspension of glutathione-free acetone-yeast were treated with 0.2 cc. of a 1:40 rat kidney extract. The glyoxalase activity was determined immediately, 0.1 mg. of pure glutathione being added from the side arm, all other conditions being the same as previously described.

That the reaction between glutathione and kidney antiglyoxalase does not involve the —SH group is indicated by the results shown in Table V. Pure glutathione (10 mg.) was incubated at 5° with the indicated amounts of acetone-ether-dried pig kidney, in a total volume of 10 cc. At intervals, portions were removed and deproteinized with an equal volume of 4 per cent sulfosalicylic

TABLE IV  
*Effect of Enzyme Concentration on Antiglyoxalase Action*

Washed acetone-yeast	CO <sub>2</sub> in 20 min.		Glyoxalase activity compared to control
	Control	Plus kidney	
cc.	c.mm.	c.mm.	per cent
0.25	54	43	80
0.50	97	82	84
0.75	145	119	82
1.0	179	147	82

TABLE V  
*Action of Kidney Antiglyoxalase on Pure Glutathione*

Dried kidney	Time of incubation	Loss of glyoxalase activity	Iodate titration*
mg.	hrs.	per cent	cc.
250	1	47.7	1.67
	5	63.8	1.69
	24	100.0	1.62
125	1	33.5	1.68
	5	45.9	1.75
	24	71.2	1.75
62.5	1	24.3	1.70
	5	26.8	1.64
	24	40.2	1.75

\* The value for the glutathione originally present was 1.70 cc. of 0.001 N iodate.

acid. 1.0 cc. of the filtrate (corresponding to 0.5 mg. of the original glutathione) was titrated for sulfhydryl by the iodate procedure of Woodward and Fry (14). 0.2 cc. of the sulfosalicylic acid filtrate (corresponding to 0.1 mg. of the original glutathione) was added to 0.4 cc. of 10 per cent washed acetone-yeast, and the glyoxalase activation determined in the usual manner. Simul-

taneously, a control activation was run, the same amount of enzyme and 0.1 mg. of pure glutathione being used. The results show not only that kidney antiglyoxalase acts on pure glutathione so that the latter loses its ability to activate glyoxalase, but also that no loss of titratable  $-SH$  occurs. The effect must be on some other part of the molecule.

Similar results were obtained when fresh kidney extracts were incubated with pure glutathione, the latter being determined by Mason's (15) method. Thus when 5 mg. of glutathione were allowed to stand for 10 minutes at room temperature with 10 cc. of 1:5 rat kidney extract in a total volume of 25 cc., no loss of sulfhydryl had occurred (found, 9 per cent above theoretical). Yet when a portion of the incubation mixture was deproteinized with sulfosalicylic acid (this treatment removes the antiglyoxalase), it was found that the resulting filtrate had lost completely its ability to activate glutathione-free glyoxalase.

These results are of interest with regard to the question of the mechanism of glyoxalase activation by glutathione. In a previous paper (12) evidence was presented that the glutathione molecule combines with methylglyoxal through the sulfhydryl group, the resulting complex then acting as the true substrate for the enzyme. The data just presented show that kidney antiglyoxalase inactivates glutathione at some other point than at the  $-SH$  group. It follows that there exist in the glutathione molecule at least two active centers which are essential for glyoxalase activation. This idea is in harmony with the fact that no sulfhydryl compound besides glutathione is known which has any activating effect on glyoxalase.

*Pancreas*—Following the general procedure used in the work with kidney, it has been found that the antiglyoxalase effect of pancreas preparations is somewhat more complicated than that of kidney, and that probably two factors are involved. The chief evidence for this belief is presented in Table VI, which shows the relation between loss of glutathione and loss of glyoxalase activity with time, in reaction mixtures containing liver glyoxalase and fresh pig pancreas extracts. Except for the substitution of pancreas for kidney extract, this experiment is identical with that recorded in Table II. The results are expressed as per cent loss of glutathione (manometric) and of glyoxalase activity, based on the



values for the original liver control in the absence of inhibitor. As in the case of the kidney experiment (Table II) this liver control represents the glyoxalase activity and glutathione content of 0.4 cc. of liver extract (no pancreas present) as determined at the time the incubation mixtures of liver plus pancreas were set up. Included also is a calculation of the per cent loss of glyoxalase activity based on the liver plus pancreas control. This control represents the glyoxalase activity (c.mm. of  $\text{CO}_2$  in 20 minutes) of the liver plus pancreas reaction mixtures as determined immediately after mixing, but before incubation. This value will be

TABLE VI

*Relation between Loss of Glutathione and Inhibition of Liver Glyoxalase by Pig Pancreas Extract*

Volume of pancreas extract per cc. of liver extract	Time of incubation	Loss of GSH (manometric)	Glyoxalase activity loss based on	
			Liver control	Liver plus pancreas control
cc.	hrs.	per cent	per cent	per cent
1.0	0		69.8	
	3	44.2	80.7	35.8
	5	59.0	86.9	53.1
0.5	0		50.3	
	3	29.5	64.2	28.3
	5	44.2	70.6	40.8
0.25	0		36.2	
	3	21.0	48.6	19.7
	5	31.1	54.6	28.7

smaller than that of the liver control because of the inhibition of glyoxalase activity produced by the antiglyoxalase during the 20 minutes required to make the determination.

It will be seen from Table VI that the glyoxalase activity losses, based on the original liver control, are invariably much higher than are the corresponding glutathione losses. However, the latter agree quite closely with the activity losses which are calculated from the values for the liver plus pancreas controls determined immediately after mixing the enzyme and inhibitor solutions. Similar results were obtained with pancreatin. These findings indicate that pancreas preparations probably contain

two antiglyoxalase factors, one of which acts on the glutathione as does kidney antiglyoxalase, and a second, which exerts an immediate inhibiting effect on the enzyme itself. Pancreas, in contrast to kidney, produces a very high immediate inhibition which then increases slowly. Thus 1.0 cc. of pancreas extract produced an immediate inhibition of 69.8 per cent (Table VI) which increased to 86.9 per cent in 5 hours. But 1.56 cc. of kidney extract produced an immediate inhibition of only 24.2 per cent (Table II) which however increased to 79.7 per cent in 5 hours. It will be observed that much larger amounts of pancreas, than of kidney, must be used to obtain the same degree of inhibition. It is quite possible that we are not dealing here with a specific inhibiting factor acting on the enzyme, but that the large quantity of protein which is introduced with the pancreas preparation interferes in some physical manner with the enzyme reaction.

Despite the evidence just presented, we have thus far not been able to demonstrate directly the presence in pancreas of an inhibiting factor acting on the enzyme. As with kidney, the inhibition produced by pancreas is independent of the amount of enzyme present, provided constant amounts of inhibitor and glutathione are present. Likewise when glutathione-free enzyme is incubated with pancreas extract, and the glyoxalase activity with added glutathione determined at intervals, there is no increase in inhibition up to 22 hours (compare Table III which shows the same effect for kidney). It is evident that if a second factor, acting on the enzyme, is present in pancreas, its total effect must have been exerted within the 20 minutes required to make the immediate glyoxalase activity determination. It appears certain that most of the inhibition obtained with pancreas after the first 20 minute period is due to an antiglyoxalase which resembles that of kidney in acting on glutathione.

That this latter factor in pancreas exerts its effect on some other part of the glutathione molecule than on the sulfhydryl group is shown by Table VII. Reaction mixtures were prepared containing the indicated amounts of pancreatin per 0.1 mg. of pure glutathione. The mixtures were incubated at 5° and at intervals portions were removed and deproteinized with sulfosalicylic acid. One portion of the filtrate was titrated for sulfhydryl with iodate,

while a second portion was added to glutathione-free acetone-yeast and the activating effect determined exactly as described for kidney (Table V). The deproteinization removes both inhibiting factors present in pancreas.

These results show that pancreatin acts on pure glutathione, destroying its ability to activate glyoxalase. Girsavicius had previously found no effect of pancreatin on pure glutathione except in the presence of fresh tissue extracts. Although the results are not as clear cut as with dried kidney, they do definitely indicate that, as with kidney, most of the destructive effect on glutathione does not involve action on the sulfhydryl group. Only small

TABLE VII  
*Action of Pancreatin on Pure Glutathione*

Pancreatin per 0.1 mg. glutathione	Time of incubation	Loss of glyoxalase activity	Loss of sulfhydryl
mg.	hrs.	per cent	per cent
12.5	4	30.8	7.3
	24	71.3	17.7
6.25	4	17.8	5.5
	24	44.6	8.6
3.13	4	14.0	0.0
	24	22.7	4.8

losses in sulfhydryl, due probably to autoxidation, occur compared to the losses in activating effect.

#### SUMMARY

1. Kidney tissue of rat, rabbit, pig, and horse contains a powerful inhibitor of the enzyme glyoxalase. A method is described for measuring the quantity of inhibitor present.

2. The inhibition produced increases with time. The inhibitor exerts its effect by destroying the coenzyme glutathione, the enzyme itself being unaffected. The action on glutathione does not involve destruction of the sulfhydryl group.

3. Evidence is presented that the antiglyoxalase effect of pancreas may be due to two factors, one of which acts on the enzyme, and a second which resembles the kidney inhibitor in acting on the glutathione but not on the sulfhydryl group.

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# THE EFFECT OF INGESTED COD LIVER OIL, SHARK LIVER OIL, AND SALMON OIL UPON THE COMPOSITION OF THE BLOOD AND MILK OF LACTATING COWS

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The feeding of cod liver oil to a lactating cow is followed by a reduction of the fat percentage in the milk. Golding *et al.* (1), who described this effect, found the fat was decreased by about 30 per cent. Results from several other laboratories have confirmed this original observation (2, 3). The phenomenon is so consistent, and the decrease in fat percentage is so large, that few animals need be employed in such studies. Golding and Zilva (4) found that a daily feeding of more than 56 gm. of cod liver oil per cow was necessary to produce either a lowering of the fat per cent or an increase in the fat-soluble vitamins of the milk.

Nothing is known concerning the fractions of cod liver oil that are responsible for this interference in fat secretion except that Golding (5) reported that the non-saponifiable fraction from the oil caused no lowering of the milk fat. The changes that take place within the body that may lead to this decrease in the secretion of milk fat are also unknown. In an earlier report the muscular degeneration that accompanies the feeding of cod liver oil to herbivora was described by us (6). The non-saponifiable fraction of the cod liver oil proved much less effective in producing the lesions than the entire oil. This suggested the possibility that the toxic agent here involved and that affecting fat percentage might be similar, or at least that they occurred in the triglyceride fraction in both cases.

The purpose of the present study was to determine first if the

chemical composition of the blood is changed in any way that may afford clues to the pathological changes induced by feeding cod liver oil. In the second place it was desired to detect any differences between the mammary and jugular blood samples during the oil feeding in contrast to the periods without oil. A third purpose was to test some other fish oils to determine if all exercise this unfavorable influence upon fat secretion. In the fourth place it was of interest to determine which fraction of cod liver oil carried the toxic agent. Finally, it was desired to learn if this effect of cod liver oil upon the composition of milk could be counteracted by feeding fresh green grass, since we had already observed that larger amounts of cod liver oil were required to develop heart lesions in goats upon pasture than in those fed synthetic diets.

*Preliminary Experiment*—Two Holstein cows producing 13 to 14 kilos of milk daily were selected for preliminary trials to determine the effect of cod liver oil feeding upon the composition of blood and milk. Samples of blood were taken from both the mammary and jugular veins at weekly intervals during a period of 4 weeks while the cows were fed the usual herd ration. During the next 4 weeks each cow was fed the same ration plus 168 cc. of cod liver oil daily. Blood samples were taken at weekly intervals again.

The blood was centrifuged and the plasma used for the determination of inorganic phosphorus (7), lipid phosphorus (8), total fatty acids (9), iodine number of the plasma lipids (10), and reducing value (11). Aliquots of milk were taken at each milking and pooled weekly for the determination of fat.

The blood sugar ranged in value from 35 to 65 mg. during the period without oil and from 37 to 56 during the 4 weeks with oil. Likewise the values for lipid P and inorganic P showed no changes from normal as a result of the cod liver oil feeding. The total lipids in the case of one cow varied from 446 to 585 mg. and in the other from 616 to 869 mg., with no change during the oil feeding period. The iodine numbers of the plasma lipids ranged from 84 to 98 during the period without oil and from 101 to 116 during the cod liver oil feeding. The increase in the iodine number of these blood lipids occurred the 1st week of the oil feeding. The fat per cent of the milk dropped the 1st week of oil feeding but did not attain the minimum value until the 2nd week.

The high iodine numbers of the blood lipids during the oil feeding period indicated the more unsaturated character of the circulating lipids which were absorbed from the oil. We have made similar observations on goats (unpublished).

The total plasma lipids, the sugar, and the lipid phosphorus values were consistently higher in the jugular than in the mammary vein. The inorganic phosphorus values showed no consistent change, contrary to the observation of Meigs *et al.* (12) that this constituent is higher in mammary blood. The present as well as our earlier findings indicate that the lipid phosphorus rises and falls in bovine blood in quite a constant relation to the total lipids.

The rapid drop of the fat percentage of the milk even in the 1st week after feeding cod liver oil, and the attainment of the minimum values the 2nd week, indicated that a period of 2 weeks afforded an adequate time for observing the effect of such oils. In the present case the milk collections were continued for 2 weeks after the oil feeding ceased. The fat percentage of the milk was back to normal the 2nd week.

*Second Experiment*—Five cows of moderate production were used in the second experiment. These consisted of three Jerseys (Cows J-1, J-2, J-3), one Holstein (Cow H), and one Guernsey (Cow G). These cows were used in two groups, started at different times. On the basis of the preceding experiment alternate test periods of 2 weeks were employed. The oil was fed daily in addition to the herd ration of grain, hay, and silage for 2 weeks, after which the cows were allowed to recover for 2 weeks upon the herd ration alone before another oil feeding period started.

The order of feeding the oils is shown in Fig. 1. During the oil feeding periods 168 cc. of the oil were fed daily by mixing it with a small amount of molasses and mixing this with the silage fed the cows each day. One cow refused to eat the feed with the cod liver oil and this cow was fed by drenching. The cows were weighed regularly but made no significant changes in weight in the course of these experiments.

Shark liver oils from two sources were employed. One was a mixed sample provided through the courtesy of Mr. R. P. Nichols. It was a mixture from various shark species captured at Key West such as sawfish, nurse, and hammerhead. Before this experiment



these samples were all assayed in our laboratory individually, according to species, and found to be as rich in vitamin A as the better grades of cod liver oil. Another sample of shark liver oil was rendered for us at Jacksonville through the courtesy of F. P.

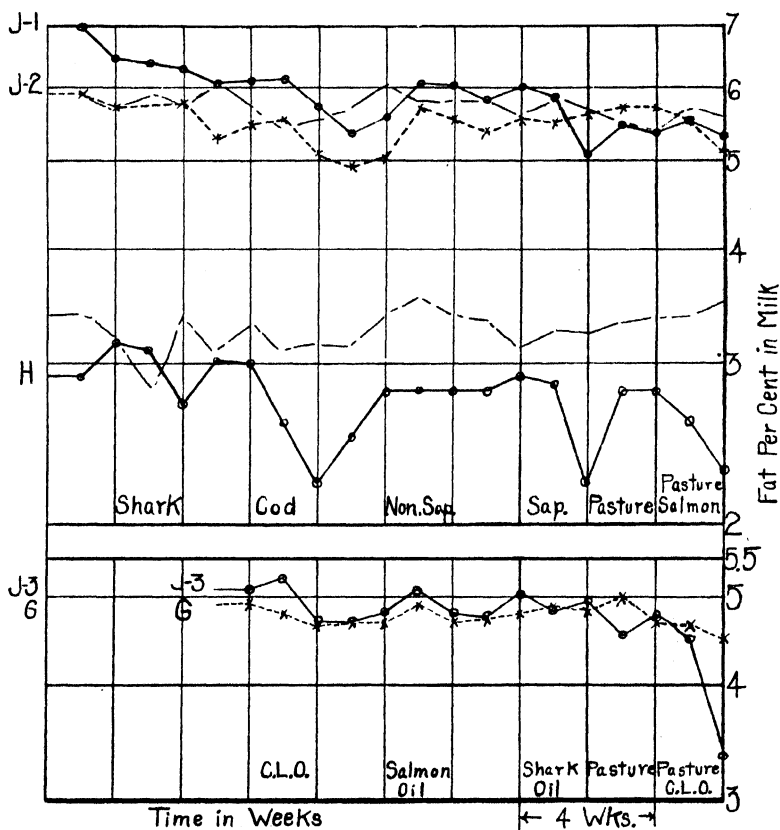


FIG. 1. The effect of feeding oils upon the fat per cent in the milk. The thin broken lines show the mean variations in the fat per cent of normally fed Jersey and Holstein cows during the same period.

Roumillat. The Key West sample was used in the case of Group I and that from Jacksonville for Group II.

► The animal grade of cod liver oil produced by the E. L. Patch Company was used in all trials. The cod liver oil fractions fed

to Group I during the 11th to 12th and 15th to 16th weeks were prepared by saponification of 8 kilos of cod liver with alcoholic potassium hydroxide in the usual manner. The non-saponifiable fraction was removed by six extractions with ethyl ether. The fatty acids were extracted with ether after acidification of the soaps with hydrochloric acid. After being washed with water the fatty acids were fed as such, while the non-saponifiable fraction was diluted with Wesson oil until it equalled the original volume of the cod liver oil. The salmon oil was a product from the Alaska Packers Corporation, Alameda, California.

At the beginning of the 17th week the cows were turned to pasture. The final oil feeding period of the 19th and 20th weeks was included to determine if fresh, green material tended to counteract the adverse effects of fish oils.

Samples of blood were taken simultaneously from the mammary and jugular veins of each cow of Group I on the last day of each week. Porcher and Maynard (13) found that the time of day at which blood samples of cows were taken made no difference in regard to the lipid level. However, we followed the practice of sampling on alternate weeks at 8 a.m. following the morning, and at 11 a.m. preceding the noon milking. The cows in Group I proved to be unusually docile and did not fight the insertion of needles into the two veins simultaneously by different workers. In the present study the plasma was used for the determination of non-protein nitrogen, total lipids, lipid phosphorus, and inorganic phosphorus, as well as true glucose and total reduction. For the last two determinations the procedure of Somogyi (14) was followed. These blood samples were taken for the first 14 weeks. No blood studies were made on Group II.

Milk samples were taken on all cows at each milking. Aliquots were pooled at weekly intervals. The per cent of fat was determined by both the Babcock and Roesse-Gottlieb methods. The iodine number of the milk fat was determined by the Hanus method. The lipid phosphorus of milk was determined by the same method used for that of blood plasma except that both hydrogen peroxide and perchloric acid were used for the oxidation.

The changes in the fat percentage of the milk during the entire period for all five cows are shown in Fig. 1. In every case cod liver oil lowers this percentage. To show that these changes are

not fortuitous, two curves for comparison are included in thin broken lines. One of these is based upon the mean fat per cent in the milk of ten Holstein cows from the same herd during the same period. The other is a similar curve for ten Jerseys.

In no case was there a lowering of the fat percentage as a result of feeding the non-saponifiable fraction, but in two of the three cases the fatty acids of cod liver oil produced the same drop as the original cod liver oil. The effect of feeding salmon oil and shark liver oil upon the milk fat per cent is questionable. If these oils have any effect it is slight compared to cod liver oil and also less consistent. There is no evidence that the feeding of pasture grass is able to counteract this effect of cod liver oil.

In all cases milk yield followed a normal course and thus the fat yield reflected the changes in fat percentage. The ingestion of the oils in all cases caused a rise in the iodine number of the milk fat. This rise was greatest during the pasture period.

The lipid phosphorus was determined in each of the weekly milk samples. In the course of 20 weeks these milk lipid P values in terms of mg. per 100 cc. varied as follows for the different cows: 6.9 to 9.2 for Cow J-1, 6.2 to 8.2 for Cow J-2, 4.9 to 8.7 for Cow J-3, 5.4 to 10.0 for Cow H, and 5.3 to 9.1 for Cow G. A comparison of these data with those for fat percentage in Fig. 1 indicated that the lipid phosphorus of the milk did not fluctuate with the total lipids. Furthermore, no consistent relation could be traced between the feeding of cod liver oil and this constituent of the milk. The breed of cow seems to make no difference in the amount of lipid phosphorus that is secreted, although our data are much too meager to serve as more than an indication. A spot diagram, involving about 90 values for each component, as well as graphs, indicated no correlation between the fat percentage of milk and the lipid phosphorus. Holm *et al.* (15) found about the same amount of phospholipids in three different samples of milk ranging in fat from 2.5 to 4.3 per cent. The phospholipids of the milk are much less in relation to the total lipids than they are in the blood although the amount per unit volume is not very different in the two cases.

In the three cows studied the true glucose of the jugular blood in mg. per 100 cc. proved to be  $34.4 \pm 1.3$ ,  $30.7 \pm 1.0$ , and  $36.6 \pm 2.1$ , while the values for the mammary blood were  $27.9 \pm 1.4$ ,

25.3  $\pm$  0.8, and 31.2  $\pm$  1.2, respectively. The other reducing substances of the blood in the same cases were for the jugular 31.5  $\pm$  0.75, 32.1  $\pm$  1.0, and 32.0  $\pm$  2.4. For the mammary they were 32.9  $\pm$  2.1, 30.1  $\pm$  1.4, and 30.1  $\pm$  1.7, respectively. In each case these mean values were based upon fourteen samples of blood taken at weekly intervals as described previously. These data indicate clearly that the mammary gland removes true glucose from the blood. If any of the other reducing substances are removed the amount is too small to be detected with certainty from these data. These findings accord with those of Blackwood and Stirling (16).

The mean values in mg. per 100 cc. for the total plasma lipids of the mammary blood were 453.1  $\pm$  5.8, 490.8  $\pm$  6.2, and 586.7  $\pm$  5.1, while those for the jugular samples were 470.9  $\pm$  4.7, 506.2  $\pm$  4.0, and 602.3  $\pm$  6.3, respectively. The corresponding mean values for the lipid P were 8.36  $\pm$  0.13, 9.0  $\pm$  0.12, and 8.88  $\pm$  0.37 for the mammary, and 8.70  $\pm$  0.12, 9.14  $\pm$  0.14, and 9.54  $\pm$  0.41 for the jugular plasma. The total lipids of the mammary plasma are significantly lower than those of the jugular blood. The lipid phosphorus values for the mammary blood are also lower, but the differences are not significant, statistically. Blackwood and Stirling (17) have recently shown that the lipid phosphorus is somewhat lower in mammary than in jugular blood for both dry and lactating cows, but not lower than in arterial blood which may be considered more representative of the supply to the gland.

The mean values in mg. per 100 cc. for the inorganic P of the mammary plasma samples were 6.06  $\pm$  0.23, 5.60  $\pm$  0.11, and 6.25  $\pm$  0.16, while those for the jugular were 6.26  $\pm$  0.32, 5.36  $\pm$  0.11, and 6.05  $\pm$  0.59, respectively. These values were obtained from weekly samples taken for 14 consecutive weeks.

As is shown by these data, no significant differences were found in the inorganic phosphorus of the mammary and jugular samples, contrary to the finding of Meigs and coworkers (12). The data reveal no relationships between the amounts of the blood lipids or the P values and the periods of ingestion of the fish oils. This indicates that cod liver oil exerts its effect directly on the mammary gland, rather than indirectly by alterations in the composition of the blood. Thus we have no clues at the present time concerning possible changes that occur in animals prior to the onset of the

pathological conditions of the muscles, liver and kidneys, and other organs, which develop when cod liver oil is fed for a period of weeks.

#### SUMMARY

Five cows were fed cod liver oil, shark liver oil, and salmon oil for periods of 2 weeks with periods without oil between. The composition of both the blood and milk was studied in the case of three of the cows and the milk only in the other two. The usual lowering of the milk fat as a result of the cod liver oil feeding was observed, but the effect of the other two oils was so much less and so inconsistent that it is questionable whether they act like cod liver oil. The non-saponifiable fraction of cod liver oil has no effect upon the milk fat, while the triglycerides seem to carry the injurious fraction. This suggests the possibility that the fraction responsible for the production of muscle lesions in herbivora, when cod liver oil is fed, may be the same as that which depresses fat secretion.

Blood samples were taken simultaneously from the mammary and jugular veins, at weekly intervals. No relation was found between the oil feeding and the composition of the blood other than the increase in the iodine number of the plasma lipids which was studied in only the preliminary trials. These data indicate that the mammary gland takes true glucose from the blood rather than other reducing substances. Furthermore, the total lipids of the mammary plasma were consistently lower than those from the jugular, suggesting that the gland removes lipids in some form from the blood. However, neither the lipid phosphorus nor the inorganic phosphorus values of the plasma furnished any evidence in support of the theory that phospholipids are the blood precursor of milk fat.

The feeding of pasture grass to cows does not counteract the effect of cod liver oil in lowering fat percentage. During the pasture feeding the iodine number of the milk fats attains its highest value if the fish oils are fed in addition to pasture grass.

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## THE PREPARATION OF GLUTAMINE\*

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(Received for publication, January 4, 1935)

Glutamine, the amide of glutamic acid, although widely distributed in nature, is a rare and relatively little known substance. Its significance in plant physiology is, however, equally as great as that of the well known lower homologue asparagine, but the difficulty of obtaining supplies of the substance has greatly interfered with investigations of its properties.

Interest in glutamine as a plant constituent has been stimulated recently by the publication by Chibnall and Westall (1) of an indirect method to determine it in plant tissues. Greenhill and Chibnall (2) have found it to be produced in such quantities by perennial rye-grass, when this is heavily fertilized with ammonium sulfate, that an exudate which dries on the upper parts of the leaf blades consists largely of crystalline glutamine. The tomato plant also has been observed in this laboratory (3) to store large quantities of glutamine under analogous conditions. We have accordingly studied the method of preparation of this substance with the view to rendering it more readily available.

A survey of the literature suggested that the most suitable tissue to employ for the preparation of glutamine in quantity is the root of the common beet (*Beta vulgaris*). The method of preparation originally described by Schulze and Bosshard (4) is, in outline, extremely simple and can hardly be improved upon; the modifications suggested below have chiefly to do with the application of modern apparatus and technique.

From 5 to 6 kilos of trimmed and washed beet roots are ground

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

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in a meat chopper and the pulp is pressed at the hydraulic press. The residue, enveloped in a bag of cheese-cloth, is suspended in ether for half an hour in order to cytolize the cells (5), is then again pressed, and washed twice with a small volume of water, being pressed each time. If desired, an aliquot may be removed for determination of the glutamine content by the indirect method, and the extract (about 8 liters) is then treated with a small excess (about 500 cc.) of basic lead acetate reagent (6), and the precipitate is removed and washed twice at the centrifuge. The pale yellow solution is finally filtered perfectly clear through a pad of paper pulp. A small excess (500 to 600 cc.) of mercuric nitrate reagent<sup>1</sup> is added (a drop of reagent added to a 10 cc. sample of centrifuged fluid should produce no turbidity in 5 minutes) and the solution is neutralized to litmus paper by the addition of 10 per cent sodium hydroxide solution (300 to 400 cc.). The white precipitate settles promptly, and it is usually convenient to allow the preparation to stand overnight at this point; the mother liquor may then be siphoned off and the precipitate washed twice by decantation, otherwise it is necessary to wash it by centrifugation.

The precipitate is collected at the centrifuge, suspended in about 3 liters of water in a filter flask, and 2 cc. of 10 per cent (by volume) sulfuric acid are added. It is then decomposed by hydrogen sulfide delivered under slight pressure with constant agitation (machine), and the mercuric sulfide is centrifuged off and washed twice with water. The colorless solution is distributed between two 5 liter ring-necked flasks, and is concentrated *in vacuo*, with use of still heads equipped with vapor coolers (7), at a water bath temperature of 60°<sup>2</sup> for about 20 minutes in order to remove the hydrogen sulfide, and is then neutralized to litmus paper with ammonium hydroxide.<sup>3</sup> Concentration *in vacuo* at 60° bath tem-

<sup>1</sup> To 160 cc. of concentrated nitric acid add 220 gm. of mercuric oxide slowly with stirring, then add 160 cc. of water and boil gently with reflux for 3 to 4 hours or until the oxide is dissolved, cool and add sufficient N sodium hydroxide (approximately 250 cc.) to produce a very faint opalescence, dilute to 1000 cc. and filter, and preserve in a dark bottle.

<sup>2</sup> Glutamine is rapidly hydrolyzed in water solution at higher temperatures.

<sup>3</sup> The use of ammonia is necessary; in our experience glutamine will not crystallize from mother liquors neutralized with magnesium, sodium, or lithium hydroxides.

perature is then continued until the total volume has been reduced to about 800 cc. The solution is then filtered through a layer of paper pulp to remove traces of mercuric sulfide, and vacuum concentration is continued in a 1 liter ring-necked flask until a thick sludge of glutamine crystals has separated. The flask is removed from the still, the air inlet tube is washed with a few cc. of hot water (60°), and the contents of the flask are warmed to 60°; this should not redissolve all the glutamine. The volume of fluid is roughly estimated, and twice this volume of alcohol is slowly added with agitation. After being chilled overnight; the crystals are filtered off, washed twice with 80 per cent alcohol, and then with absolute alcohol and, if the weight is desired, are dried *in vacuo* over sulfuric acid. For recrystallization the crystals are

TABLE I  
*Glutamine Prepared from Beets*

Glutamine in extract (indirect analysis)	Crude crystals	Recrystallized	Yield	N content
<i>gm. per kg. fresh tissue</i>	<i>gm. per kg. fresh tissue</i>	<i>gm. per kg. fresh tissue</i>	<i>per cent</i>	<i>per cent</i>
1.937	1.832	1.583	81.8	18.6
2.793	2.575	2.290	82.0	18.9
3.009	2.788	2.554	84.9	18.8
1.733		1.336	78.9	

dissolved in 10 times their weight of water at 60°, and are treated with a little norit at this temperature for 5 to 10 minutes. After filtration, 1 volume of absolute alcohol is added to the hot solution, and the beaker is allowed to stand until cold; a second volume of absolute alcohol is then added, and crystallization is allowed to continue overnight in the refrigerator, the mother liquor being thoroughly stirred once or twice to prevent supersaturation. The crystals are filtered, washed, and dried as before. The yield depends on the richness of the tissue employed, and this may vary from 1.7 to 4.5 gm. or even more per kilo of roots as determined by the indirect method on the extract. Higher yields are usually to be obtained from beets that have been in storage for some time than from fresh plants. In any case the yield of recrystallized material should be approximately 80 per cent of the quantity of glutamine present in the tissue.

Table I shows data from several preparations carried out as described. The products melted at or near  $182^{\circ}$ , and were slightly low in nitrogen content. A number of preparations were combined and crystallized once more. The resulting crystals contained 19.0 per cent of nitrogen (theory 19.18), 9.46 per cent of amide nitrogen (theory 9.59), and melted at  $185-186^{\circ}$  (short stem thermometer). Bergmann, Zervas, and Salzmann's synthetic *d*-glutamine (8) melted at  $184-185^{\circ}$ .

#### SUMMARY

The preparation of glutamine from the root of the common beet is described. The method employed is essentially that of Schulze and Bosshard but is modified in detail. Yields of the order of 80 per cent of the glutamine content of the tissue are readily obtained.

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# **A BIOCHEMICAL STUDY OF THE FERMENTATION OF RARE SUGARS BY MEMBERS OF THE COLON AND AEROGENES GROUPS OF BACTERIA**

## **II. CELLOBIOSE**

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(Received for publication, December 27, 1934)

Most of the quantitative work on the fermentation of sugars and higher polyhydric alcohols has been done on the more common members of these groups of compounds. An investigation is in progress in this laboratory on the metabolic products which are produced by members of the colon and aerogenes groups of bacteria in media containing some of the rarer sugars and alcohols, the results of the work with trehalose and *d*-sorbitol having already been reported (1, 2). The investigation covered in this communication gives the results when cellobiose (cellose) was used as the carbohydrate constituent of the medium. A number of investigators (3-7) have reported on the qualitative fermentation of this sugar.

The methods and procedures were essentially those employed in the previous studies (1, 2). For the quantitative determination of the products of fermentation, a medium was used containing 5 gm. of cellose and 8 gm. of Bacto-Nutrient broth in 1 liter of water. This medium was adjusted to a pH value of 7, and 100 cc. amounts were placed in 250 cc. flasks and sterilized at a pressure of 10 pounds on each of 2 consecutive days. In addition, 50 cc. amounts were placed in Smith fermentation tubes and properly sterilized. Each fermentation tube was previously fitted at the top of the gas arm with a small bore glass tube which was sealed with a short piece of rubber tubing containing a plug of solid glass rod. This arrangement facilitated the transfer of the gas.

Determinations of pH values were made at frequent intervals with the hydrogen electrode potentiometer in media containing

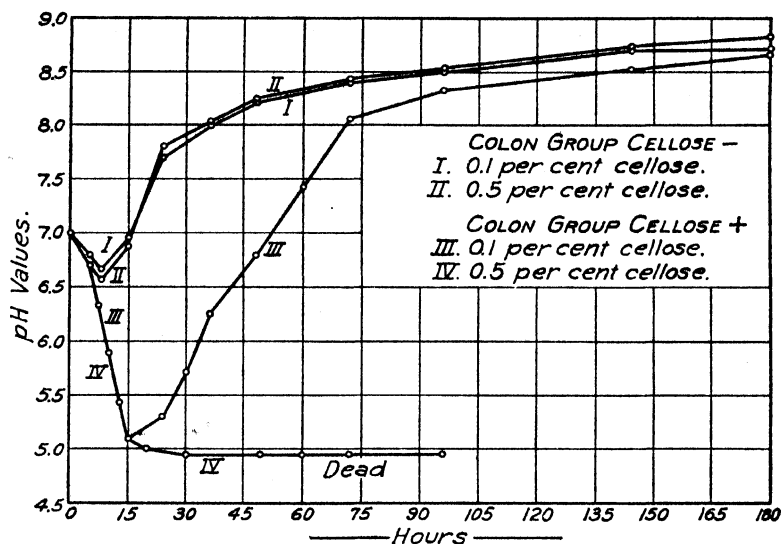


FIG. 1. Curves showing the change in pH values when cellulose-positive and cellulose-negative organisms of the *Escherichia* group are grown in cellulose media.

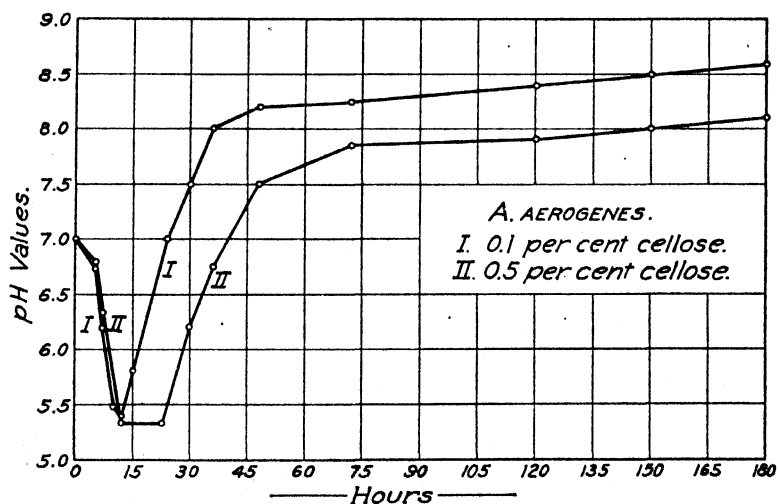


FIG. 2. Curves showing the change in pH values when *Aerobacter aerogenes* is grown in cellulose media.

0.1, 0.5, and 1.0 per cent cellose. Various strains of four organisms were used: *Aerobacter aerogenes*, *Escherichia coli*, *Escherichia communior*, and *Escherichia neapolitana*. Some of the cultures of the *Escherichia* group produced gas in cellose media and others gave no gas production. The values obtained with the medium containing 1 per cent sugar were about the same as those with the medium containing 0.5 per cent sugar. The average values for

TABLE I  
Fermentation Products in Cellose Medium

Culture No.....	<i>Escherichia coli</i>		<i>Escherichia communior</i>		<i>Escherichia neapolitana</i>	<i>Aerobacter aerogenes</i>		<i>Aerobacter cloacae</i>
	82	71	29	304	445	257	17	59
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
CO <sub>2</sub> *.....	1.5	None	5.3	None	None	9.1	5.0	5.8
H <sub>2</sub> *.....	4.5	"	10.5	"	"	11.9	7.5	8.0
CO <sub>2</sub> /H <sub>2</sub> .....	0.30		0.50			0.77	0.67	0.73
Volatile acid†.....	10.9	13.3	14.2	9.0	10.8	9.8	12.2	12.5
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Formic " ‡.....	2.5	1.2	2.2	1.0	1.8	1.6	1.5	1.2
Acetic ".....	62.2	78.2	82.3	52.7	62.4	56.7	71.2	73.4
Succinic ".....	5.8	6.9	15.3	7.0	5.3	8.7	8.6	14.2
Lactic ".....	14.9	15.2	27.2	23.5	21.6	21.2	28.4	32.6
Alcohol.....	402.0	322.0	298.0	83.0	112.0	282.0	133.0	163.0
Reducing sugar after hydrolysis as glucose.....	None	672.0	None	657.0	736.0	None	None	None
Ratio, volatile/non-volatile.....	3.1	3.6	2.0	1.8	2.4	1.9	2.0	1.6
Acetic/succinic.....	10.7	11.3	5.4	7.5	11.8	6.5	8.3	5.2
" /lactic.....	4.2	5.1	3.0	2.2	2.9	2.7	2.5	2.3

\* Volume formed in a Smith fermentation tube.

† Amount of 0.1 N alkali used to neutralize the acid from 1 gm. of sugar.

‡ All of the values in mg. are on the basis of 1 gm. of cellose.

three strains of *Aerobacter aerogenes* and for four strains of *Escherichia* are given in Figs. 1 and 2. The pH values with both groups of organisms in the medium containing 0.1 per cent sugar reached a minimum in about 12 hours, and then the values gradually increased until a maximum pH value of around 8.5 was reached. The minimum was somewhat lower in the medium containing 0.5 per cent sugar, and the acidity which developed killed the organ-

isms in a number of cases. With the cellulose-negative organisms, the pH values were not as low on the acid side as with the cellulose-positive strains, a value of 6.6 being the lowest recorded.

The medium contained in the flasks and the Smith fermentation tubes was inoculated and incubated for 72 hours at 37°. The fermentation products were analyzed and the results are given in Table I.

The amounts of the products formed with the two groups of bacteria show very little difference. The aerogenes group, on the whole, showed larger amounts of succinic and lactic acids, and the gas ratios were greater. The acid ratios for the two groups of bacteria were very nearly the same. With the cellulose-positive organisms of the *Escherichia* group, all of the cellulose was used up during the 72 hours, whereas on the other hand, there was considerable unused sugar remaining when the cellulose-negative organisms were grown in the same medium. The difference in the products formed by the colon and aerogenes groups in cellulose media is not nearly so characteristic as was shown by these groups of bacteria in media containing trehalose (1).

#### SUMMARY

1. The fermentation products produced by members of the *Escherichia* and *Aerobacter* groups of bacteria in cellulose media have been determined.

2. The progressive changes in pH values during fermentation have been studied.

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# THE DETERMINATION OF THE COLLOIDAL OSMOTIC PRESSURE IN BLOOD SERUM AND SIMILAR FLUIDS

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The importance of the colloidal osmotic pressure in biological fluids is becoming increasingly recognized, but difficulties in the older methods as yet prevent their general use in inquiries for which needed information can only be obtained from colloidal osmotic pressure data.

The membrane bag arrangement used by Starling (1896) is capable of yielding admirable results (see, *e.g.*, Adair, 1925; Adair and Robinson, 1930), but it requires so much time and material that its use has been limited. The methods in greatest use are those of Schade and Claussen (1924), Govaerts (1923), also Verney (1926), and Krogh and Nakazawa (1927) (see also Wells, 1933; Hill, 1932-33).<sup>1</sup> These all require special apparatus which is not easy to construct and requires considerable skill to use.

In addition to technical difficulties, there is a further point of criticism of the methods in current use in physiological and medical laboratories in that they are based on the assumption that it is desirable to obtain a colloid osmotic pressure reading of the colloid fluid equilibrated against its own ultrafiltrate. The justification of this is based on the assumption that the effective colloid osmotic pressure in the organism is most closely estimated in this way. However, there is no valid proof that this is the case; in this laboratory we have found evidence to indicate that, in man, neither are the capillaries wholly impermeable to blood colloids nor are the colloids alone effective as osmotic agents determining the fluid balance across the capillary membrane. In other words,

<sup>1</sup> For brief descriptions of a number of methods, see Meyer (1932).



the characteristics of artificial membranes are sufficiently different from the membrane of the capillary wall to destroy the principal argument for the ultrafiltrate procedure.

There is a positive argument against the use of the ultrafiltrate procedure in that, when blood serum is used, the variability of the crystalloid composition of the blood serum may alter the relative magnitude of the Donnan effect. If one's desire is to estimate the physical state of the proteins, with regard to apparent molecular sizes, it is obviously desirable to create constant conditions of ionic strength and pH. This was one of our chief concerns in the development of the present method.

### *Method*

The colloid solution (*e.g.*, blood serum) is diluted with an equal volume of a buffer solution of constant ionic strength which is also used as external solution. The wet osmometer cup is rinsed out several times with the diluted colloid solution and then filled to a point where the stopper bearing the manometer tube (see Fig. 1) can be inserted without trapping more than a small bubble of air. The stopper is partly inserted with the upper end of the manometer tube closed with a rubber tube and clamp, and then the end of the tube is opened and the stopper forced in until the fluid stands in the capillary manometer tube higher than the expected colloid osmotic pressure. The outside of the osmometer cup is then carefully washed with the buffer solution to remove all traces of colloid which may have spilled over, and then the osmometer is immersed in the outer (buffer) fluid as shown in Fig. 1.

The equilibration is carried out in an ice box or refrigerator room in which a temperature constant to  $\pm 2^\circ$  is maintained at some level between  $0-5^\circ$ . No manipulations are required, equilibrium being attained spontaneously, owing to the small size of the osmometers and by virtue of the initial approximation of the crystalloid concentration and pH in the inner and outer fluids, before any disturbing bacterial destruction of the proteins intervenes. Sterile technique is, therefore, unnecessary. It is desirable to test the outer fluid for protein at the end of the determination, thereby assuring the acceptability of the results.

The osmometers are read after 3, 4, and 5 days, and the gross colloid osmotic pressure is calculated in mm. of  $H_2O$  as colloid osmotic pressure =  $2(h - c)$ , where  $h$  is the vertical distance in

mm. between *A* (Fig. 1) and the level of fluid in the capillary manometer tube, and *c* is the capillarity of the manometer tube, also in mm. of  $\text{H}_2\text{O}$ .

The osmometers are extremely simple, consisting of a glass ring to the bottom of which is affixed a collodion membrane (*M*, Fig. 1) and a capillary tube carrying linear graduations in mm. The simplest, and also the best, arrangement for the manometer tube is to have two marks a fixed distance apart (*A* and *B*, Fig. 1) and a paper mm. scale held in place and protected by an outer glass tube, as shown in Fig. 1.

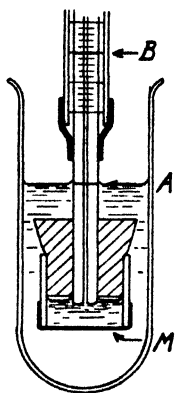


FIG. 1. The osmometer mounted as in use. *A* and *B* are fixed reference lines on the capillary, the level of the outer fluid being adjusted exactly to *A*. *M* is the collodion membrane.

The bore of the capillary tube should be about 1 mm. or a trifle less, and its capillarity determined with a diluted colloid solution similar to the fluid to be tested. The capillary tubes should always be scrupulously clean and dry before use.

The membranes are made with a procedure somewhat similar to that used by Adair and Keys (1934). A glass tube of about 3 cm. diameter is mounted to rotate slowly in the horizontal plane; then pour on three coats of collodion, proceeding smoothly from one side to the other, over a length of about 10 cm. The first coat is dried for  $1\frac{1}{2}$  minutes after pouring at about  $30^\circ$ , the second dried for 2 minutes at  $30^\circ$ , and the third for 5 minutes at  $30^\circ$  (an electric heater lamp is excellent for this). Several minutes after the last

drying, the membrane is cut and peeled off as a flat sheet, still quite soft. With the sizes mentioned here, four membranes can be prepared and accordingly the original soft sheet may be cut in quarters.

The soft membrane is laid over the end of one of the glass osmometer rings and molded over the end with the fingers, this molding being repeated several times until the membrane sticks firmly. The excess membrane is then trimmed off and a drop of fresh collodion run around the ring as a final seal. The total drying time in the air following the pouring of the third coat should be between 21 and 24 minutes, and after that the last seal is dried in the air for 1 or 2 hours with the membrane itself just dipping in water.

The collodion solution we use is prepared from 3 parts of du Pont collodion, 1 part of anhydrous ether, and 1 part of ethyl alcohol. It is allowed to stand for several days before use and thereafter keeps indefinitely. If necol collodion is used, the proper proportions are 1.5 to 2 parts of necol to 1 part of ether and 1 part of alcohol. Care should be taken not to get the membrane too thick; a thin mixture is generally better than a thick one.

These membranes should be tested, but, if they are properly made, they are always protein-tight. When not in use, they may be stored for months in 1 per cent HCl in an ice box. Before use they are rinsed in several changes of distilled water for a day or so. These membranes may be used repeatedly, provided they are never allowed to dry once they are prepared.

The suspending and outer buffer fluid may be made up to any desired pH and ionic strength. We have preferred to work with phosphate systems and have used the data of Green (1933) in preparing the solutions. Below are given the compositions, ionic strengths, and pH values for two useful solutions, the first being designed particularly for colloid osmotic pressure studies of cell content solutions, the second being perhaps somewhat better for serum protein studies.

Total ionic strength	pH	$\text{KH}_2\text{PO}_4$	KOH
		<i>mole per l.</i>	<i>mole per l.</i>
0.135	7.2	0.056	0.0395
0.150	7.4	0.058	0.0460

The glass osmometer rings we use have an internal diameter of 1.8 cm. and are usually filled to a depth of about 0.4 cm., giving a membrane surface of about 2.5 sq. cm. per cc. of fluid. Determinations in duplicate can be made with about 1.5 cc. of serum.

#### EXPERIMENTAL

In Table I are given results from a series of osmometers set up with identical serum. These results are more or less typical of the course of equilibration and the final agreement of duplicates.

Typical comparisons of hydrogen ion concentrations of internal and external solutions of human serum are given below, together

TABLE I

*Course of Equilibration and Agreement of Separate Determinations of Colloid Osmotic Pressure in Human Serum (in Mm. of H<sub>2</sub>O)*

Serum	Osmometer No.	Initial	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.
A	1	150	220	260	287	291	290
	2	170	240	270	279	286	289
B	1	400	280	276	284	284	
	2	380	322	288	296	294	

with colloid osmotic pressure values and pH values by the glass electrode. The effect of pH on the observed osmotic pressure is qualitatively similar to that reported by Marrack and Hewitt (1927).

Colloid osmotic pressure	Internal pH	External pH
<i>mm. H<sub>2</sub>O</i>		
285	7.26	7.22
295	7.39	7.36

In forty determinations of colloid osmotic pressure of human blood serum in fifteen resting subjects<sup>2</sup> we have found values for mm. of colloid osmotic pressure per gm. of protein concentration ranging from 39 to 45 with an average of 42.0. This is somewhat lower than is found with the methods of Govaerts (1923), Schade and Claussen (1924), and Krogh and Nakazawa (1927), although

<sup>2</sup> The subjects were all normal young men.

some of this difference disappears when the values are all compared at 37° by multiplication of the colloid osmotic pressure value obtained by the factor 310/(absolute temperature).

It should be remembered that the present method does not measure exactly the same thing as the other methods discussed here, but some comparison of results with the present method and with the second method of Krogh and Nakazawa may be useful. Such comparisons are made in Table II.

The present method invariably gives lower results than the Krogh and Nakazawa method. This may be due partly to the fact that, as has been generally observed (see Meyer, 1932, p. 38), with increasing protein concentration the colloid osmotic pressure

TABLE II

*Comparison of Colloid Osmotic Pressure Results with the Present Method and the Method of Krogh and Nakazawa (1927) on Human Serum*

The values are expressed in mm. of H<sub>2</sub>O.

Series No.	Concentration of serum used in present method	Present method (4°), pH 7.25	Present method corrected to 22°	Krogh and Nakazawa method (22°)
1	Undiluted	285	307	322
		284	305	327
				329
2	Diluted 1:1	270	287	330
		278	296	340

increases slightly more than might be expected. The tendency in this direction shown in Table II is general. It is also probable, however, that some of the difference is due to differences in pH and in salt concentrations leading to different Donnan effects.

## SUMMARY

A true equilibrium method for measurement of the colloidal osmotic pressure in blood serum and similar fluids is presented. Duplicate determinations can be made easily with 2.0 cc. of colloid solution and, with care, a single determination can be made on 0.5 cc. Some results with the method are presented and comparisons are made with other methods.

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# THE BEHAVIOR OF THE PLASMA COLLOIDS IN RECOVERY FROM BRIEF SEVERE WORK AND THE QUESTION AS TO THE PERMEABILITY OF THE CAPILLARIES TO PROTEINS

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It is generally agreed that the fluid balance between blood and tissue is a resultant between hydrostatic and osmotic forces not greatly different from that pictured originally by Starling (1896). The analysis of the exchanges across the capillary membrane involves, necessarily, acceptable data for both the hydrostatic pressure and the effective osmotic pressure at the site of the exchanges, or, alternatively, figures for one of these factors together with a detailed knowledge of the character of the capillary wall as a permeable membrane.

The efforts of Starling and his successors (see, *e.g.*, Schade and Claussen, 1924; Landis, 1926; Krogh, 1929) were directed toward proving that the capillary blood pressure and the osmotic pressure of the plasma colloids are so nearly equivalent to each other that the observed state of balance between blood and tissue fluid would be obtained in a passive system with a membrane permeable to everything except substances of colloidal dimensions. These efforts have been regarded as generally successful but, as Drinker and Field (1933) point out, perhaps they are quantitatively too successful in demonstrating complete equality of the two opposing forces considered.

Drinker and Field have collected a convincing body of data to show that not only thoracic duct lymph but also true tissue lymph contains a considerable amount of protein which exerts a proportionate osmotic force (Loewen, Field, and Drinker, 1931). Moreover, this tissue lymph is in constant, though slow, circulation, and Drinker and Field make it probable that this lymph is derived



directly from the true tissue fluid and is little if any different from the latter.

The presence of an appreciable colloidal osmotic pressure on the tissue side of the capillaries makes it necessary to construct a new pressure balance sheet for the fluid balance between blood and tissue. Various workers (Iversen and Johansen, 1929; Meyer and Holland, 1932; Weech, Snelling, and Goettsch, 1933) have attempted such calculations, the general form of which is given by Landis (1934) in the equation,  $CP - TP = COP_B - COP_T$ , where  $CP$  is capillary blood pressure,  $TP$  is tissue pressure, and the right-hand members of the equation refer to the colloidal osmotic pressures of the blood plasma and the tissue fluid respectively.

Aside from the fact that each of the items in the above equation is very difficult to evaluate, it should be pointed out that, as it stands, such an equation implies that the capillaries are completely impermeable to proteins and other colloids. Drinker and Field deny such colloid impermeability, believing that the protein they find in the tissue lymph is derived from filtration through the capillary wall. Landis (1934, p. 437), however, though retreating somewhat from his former position, believes that the permeability of the capillaries to protein is very slight.

With present methods the problem can scarcely be attacked profitably on the resting subject where the exchanges between blood and tissue are constant and minimal, but the exchanges produced by brief severe exercise should give favorable circumstances to observe the behavior of the plasma colloids in the normal or non-pathological organism. In a period of a minute or so destruction or manufacture of plasma protein must be considerable.

The most obvious procedure would be to compare the plasma protein concentration with the hemoglobin concentration before and immediately after the short period of exercise. This is open to the criticism that the amount of hemoglobin in the circulating blood may be inconstant, owing particularly to the reservoir action of the spleen and possibly the liver.

A second and much more promising procedure would be to investigate whether, under the stress of increased fluid movements induced by the exercise, there is any change in the average

physical dimensions of the protein particles. Such a change, if exhibited, must be due either to a sudden *in vivo* transformation of protein types, or, much more probably, to a differential loss or gain of larger or smaller particles from the blood system.

*A priori* it would seem unlikely that, if some portion leaks out through the capillary walls, the different protein molecules would escape at a rate independent of their relative sizes. Therefore it might be expected that simultaneous observation of both the blood protein concentration and the colloid osmotic pressure before and immediately after brief severe work would throw light on the ability of the capillary wall to retain protein.

Obviously, these questions of the permeability of the capillary walls to proteins must be settled in order to arrive at any satisfactory values for the fluid movements between blood and tissue, but some estimate as to probable limits of these values under these conditions may be made from the serum protein and oxygen capacity measurements.

### *Methods*

The subjects used in the present experiments were all healthy young men between the ages of 19 and 26. They all had had at least one preliminary trial on the motor-driven treadmill used. Before the experiment the subject always rested, prone, for half an hour and then a blood sample was taken, without stasis, from the antecubital vein. Immediately thereafter the subject ran on the treadmill for 1 minute at a rate of 11.6 miles per hour and at an angle of 7 per cent. In all cases this rate of work was near the limit of performance and the pulse at the end was around 200 per minute.

At the end of work the subject rested on a bed at the side of the treadmill and a second venous sample was taken. The collection of this blood sample was, on the average, completed within 2 minutes of the end of work, and when a longer time was taken the experiment was discarded.

In each case one fraction of the blood was kept from clotting by heparin; the oxygen capacity was measured on this by means of the usual Van Slyke technique, 1 cc. samples being used in duplicate. Another portion of the blood was allowed to clot at room temperature for 30 minutes, centrifuged at 2700 R.P.M. for 40

minutes, and the resulting serum used for the determination of protein and colloidal osmotic pressure. Serum was used in preference to plasma because of the possibility that anticoagulants might affect the colloidal osmotic pressure and because partial clotting, which sometimes occurs in plasma during osmotic equilibration, would completely ruin a determination.

Protein was determined from the total nitrogen obtained by the Kjeldahl method on duplicate 2 cc. portions, corrected for non-protein nitrogen as determined by Daly's (1933) modification of the method of Koch and McMeekin.

The colloidal osmotic pressure was determined by the method of Keys and Taylor (1935) in which the pH and the ionic strength of the equilibrating fluid remain constant at 7.38 and 0.135 respectively. With this method variations in the Donnan effect are minimized and the resulting measurements are, in relative terms, strictly comparable for the estimation of average colloidal sizes.

In addition to the foregoing, check determinations were made of the total dry solid and ash in both whole blood and serum.

#### EXPERIMENTAL

The principal experimental results are compiled in Table I. In the eight subjects there was, 1 minute after the work, compared to 1 minute before work, an average increase of 7.55 per cent of the initial oxygen capacity and 12.34 per cent of the initial serum protein content.

The blood concentration changes observed in these experiments must be the resultant of exchanges of fluid between blood and tissue fluid, modified by the possible loss of plasma protein from the blood stream and the possible gain of red cells from reservoirs such as the spleen. Various attempts to evaluate these modifying factors have been made, notably by Barcroft (1927, 1934) and by Dill, Talbott, and Edwards (1930). The number of variables involved and the fact that some of these factors are not accessible to direct evaluation preclude the possibility of very precise analysis, but approximate limits can be assigned for the present experiments by simple calculations.

Dill, Talbott, and Edwards (1930) showed that, in normal man, the ratio of cell volume to oxygen capacity is nearly, but not quite, constant from rest to work and recovery. They found an em-

pirical relation between pH and the cell volume to oxygen capacity ratio

$$\text{Hb per liter cells} = \frac{\text{Hb per liter blood}}{V_c - \frac{7.40 - \text{pH serum}}{30}}$$

In the present series of experiments it was not always practicable to make determinations of cell volume, but in a long series of

TABLE I

*Comparison of Blood in Rest and 1 Minute after Exhausting Work of 1 Minute's Duration*

Serum colloidal osmotic pressures are given in mm. of water, protein as gm. per 100 cc. of serum (calculated as  $6.25 \times$  nitrogen), oxygen capacity as volumes per cent. All values are arithmetic means of several determinations.

Subject No.	State	Oxygen capacity	Per cent increase	Serum protein	Per cent increase	Serum colloidal osmotic pressure	Per cent increase	Colloidal osmotic pressure per gm. protein	Per cent decrease
1	Rest	19.72		6.81		295		43	
	Work	21.12	7.10	7.60	11.60	264	-10.5	35	19
2	Rest	17.27		6.95		290		42	
	Work	18.15	5.16	7.78	11.92	297	2.4	38	10
3	Rest	20.11		7.06		305		43	
	Work	21.02	4.52	7.86	11.33	266	-12.8	34	21
4	Rest	22.45		7.00		296		42	
	Work	23.92	6.53	8.06	15.16	296	0	37	12
5	Rest	21.02		7.18		321		45	
	Work	23.30	10.82	8.42	17.26	361	12.4	43	5
6	Rest	21.19		7.20		283		39	
	Work	23.21	9.54	7.78	7.75	290	2.5	37	5
7	Rest	22.97		7.10		287		40	
	Work	23.86	3.88	7.84	10.41	291	1.4	37	8
8	Rest	20.64		7.60		316		42	
	Work	23.42	12.82	8.61	13.29	311	-1.6	36	14

experiments identical with the present series we have consistently found an average decrease of hemoglobin per unit of cell volume of 0.5 to 1 per cent. Moreover, in these experiments there was in the blood following work an average decrease of about 0.30 pH, implying a decrease of Hb per unit of cells of 0.8 per cent. For the present purposes it is sufficiently accurate, in dealing with mean values, to assume an average decrease following work of 0.8 per cent in the amount of hemoglobin per unit of cells. This value is

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used in computing the last column of Table II. The factor for conversion of oxygen capacity to cell volume in the second and third columns is taken from the nomogram of Van Slyke, Sendroy, and Liu (1932).

Since the present analysis is intended only to indicate relative magnitudes of the exchanges, we shall assume the convenient average total blood volume in rest of 6.00 liters; from the data of Brown and Rowntree (1925) and of Chang and Harrop (1928), this value cannot be very far from correct as an average for the subjects we used. It must be realized that the general picture is not influenced in the slightest by this assumption and that the

TABLE II  
Mean Values for Eight Subjects in Present Series of Experiments

	Rest	Work	Work (corrected for pH change)
O <sub>2</sub> capacity, volumes per 100 cc. blood.....	20.67	22.25	
Calculated cell volume per 100 cc. blood, %..	44.7	48.4	48.0
“ serum “ “ 100 “ “ %...	55.3	51.6	52.0
Serum protein, gm. per 100 cc. serum.....	7.11	7.99	

values in Columns 3 and 5 in Table III and in Columns 3 and 5 in Table V are exactly the same for *any* assumed resting blood volume. The limiting calculations follow.

### *Assuming Plasma Proteins Constant in the Blood Stream*

$$6000 (0.553) (7.11) = x (0.520) (7.99)$$

$$x = 5680 \text{ cc. calculated total blood volume after work}$$

Then

$$6000 (20.67) = (5680) (22.25) - y$$

$$y = 24 \text{ cc. total oxygen capacity of new cells gained, or}$$

$$\frac{24}{0.4594}$$

$$= 52 \text{ cc., volume (at resting pH) of new cells gained, or}$$

$$\frac{52}{6000 (0.447)}$$

$$= 1.9 \text{ per cent gain in new cells compared to total cells in rest}$$

### *Assuming Total Hemoglobin Constant in the Blood Stream*

$$6000 (20.67) = x (22.25)$$

$$x = 5570 \text{ cc. calculated total blood volume after work}$$

Then

$$6000 (0.553) (7.11) = (5570) (0.520) (7.99) - y$$

$$y = 4.6 \text{ gm., calculated loss of protein from blood plasma, or}$$

$$\frac{4.6}{6000 (0.553)}$$

$$= 1.4 \text{ per cent loss of protein compared to total protein in rest}$$

There are several possibilities besides those calculated above, the most obvious and acceptable of which is that there may be a simultaneous loss of some protein and a gain of some cells. The possibility that there may be large net gains of both protein and cells from outside the resting circulation cannot be entertained very seriously in view of the fact that, despite much careful work, no evidence for reservoirs of plasma proteins has ever been unearthed. All other possibilities for explaining results such as the present involve such complicated and improbable assumptions that they need not be treated here, and we believe that the true

TABLE III

*Calculated Changes in Total Blood Volume and in Total Plasma Proteins and Total Circulating Red Cells for Eight Subjects in the Present Experiments and Eight Subjects of Dill, Talbott, and Edwards (1930)*

		Total blood volume during work (at rest, 6000)	Total cells gained	Per cent of total	Total serum protein lost	Per cent of total
		(1)	(2)	(3)	(4)	(5)
Present study	Assume Hb constant	cc. 5570	cc. 0	0	gm. 4.6	1.4
	“ serum protein constant	5680	52	1.9	0	0
Dill, Talbott, and Edwards	Assume Hb constant	5679	0	0	0.7	0.4
	“ serum protein constant	5701	14	0.5	0	0

quantitative explanation of our results lies somewhere between the two calculations presented above.

Before attempting further to analyze the present data, comparison will be made with calculations from the data obtained by Dill, Talbott, and Edwards (1930). In their experiments the conditions were much the same except that the rate of work was much lower and was continued for 20 minutes. Taking the eight normal subjects in their series on which complete and comparable observations were made, we have applied the same calculations used with our subjects. The results, together with our own, are presented in Table III.

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It is obvious that both series present much the same general picture—the difference being that simple fluid movement will account for more of the changes in the longer work than in the shorter, more intense work. A similar difference was found in experiments preliminary to the main series presented here, and this difference may be of some significance and will be treated in the discussion later.

The analysis of the colloid osmotic pressure data can be developed as follows:

- Let  $a$  = "filtrable protein" concentration in gm. per 100 cc. serum  
 $b$  = "non-filtrable protein" " " " " 100 " "  
 $x$  = colloid osmotic coefficient for non-filtrable protein  
 $kx$  = " " " " filtrable protein  
 $v$  = total blood volume in deciliters  
 $c$  = colloid osmotic pressure of serum  
 $p$  = total protein concentration in gm. per 100 cc. serum  
 $s$  = cc. serum per 100 cc. whole blood  
 Subscript 1 = rest, subscript 2 = work

Then

$$(I) \quad kxa_1 + xb_1 = c_1; \quad (II) \quad kxa_2 + xb_2 = c_2$$

$$(III) \quad a_1 + b_1 = p_1; \quad (IV) \quad a_2 + b_2 = p_2$$

If we assume that the total non-filtrable protein in the blood stream remains constant, we have

$$(V) \quad b_1(s_1)(v_1) = b_2(s_2)(v_2)$$

Now (III-a)  $a_1 = p_1 - b_1$  and by substitution in equation (I):

$$(I-a) \quad kxp_1 - kxb_1 + xb_1 = c_1 \text{ or}$$

$$(I-b) \quad b_1 = (c_1 - kxp_1)/(x - kx)$$

Similarly, (II)  $b_2 = (c_2 - kxp_2)/(x - kx)$

But (V-a)  $b_1 = \frac{b_2 s_2 v_2}{s_1 v_1}$  and, by substitution in equation (I-b),

$$(I-c) \quad b_2 = \frac{s_1 v_1 (c_1 - kxp_1)}{s_2 v_2 (x - kx)}$$

On combination with equation (II-a) we have

$$(VI) \quad \frac{s_1 v_1 (c_1 - kxp_1)}{s_2 v_2 (x - kx)} = \frac{c_2 - kxp_2}{x - kx} \text{ or,}$$

$$(VI-a) \quad s_1 v_1 c_1 - s_1 v_1 kxp_1 = s_2 v_2 c_2 - s_2 v_2 kxp_2 \text{ or,}$$

$$(VI-b) \quad kx(s_2 v_2 p_2 - s_1 v_1 p_1) = s_2 v_2 c_2 - s_1 v_1 c_1 \text{ or,}$$

$$(VI-c) \quad kx = \frac{s_2 v_2 c_2 - s_1 v_1 c_1}{s_2 v_2 p_2 - s_1 v_1 p_1}$$

The value of  $kx$  may be calculated from the present data for various values of  $v_2$ . Having obtained values for  $kx$ , we may evaluate either  $b_1$  or  $b_2$  for various values of  $k$ . Clearly, the resulting values of  $b_1$ , the initial concentration of non-filtrable protein in the serum, which are greater than 7.11, the initial total protein concentration in the serum, are impossible unless we admit the existence of protein reservoirs and hence an upper limit can be assigned to  $v_2$ , the final total blood volume. The results of such calculations are presented in Table IV, again assuming for convenience that  $v_1 = 60$ ; this assumption does not, of course, affect the final results appreciably.

TABLE IV

*Calculated Values for Initial Concentration of Non-Filtrable Protein in Gm. per 100 Cc. Serum*

$k$  is the ratio between the relative osmotic activities of the filtrable and non-filtrable protein.

Value of $b_1$ (concentration of non-filtrable protein) for				
$v_2$	$kx$	$k = 4$	$k = 8$	$k = \infty$
50.0	78	4.32	3.75	3.17
53.0	110	5.89	5.03	4.39
54.0	137	6.55	5.62	4.93
55.0	193	7.40	6.35	5.56
56.0	393	8.47	7.27	6.36
56.5	1026	9.10	7.80	6.83
57.0	-1128			

Since  $b_1$  must be appreciably less than 7.11, Table IV shows, independently of hemoglobin, that  $v_2$  cannot be more than about 56.5 and, unless the non-filtrable protein is extraordinarily inactive osmotically, is probably less than 56.0. Moreover, Table IV shows that, since  $v_2$  cannot be less than about 55.6,<sup>1</sup> the filtrable protein must be, on the average, at least 5 times as active osmotically as the non-filtrable protein. Finally, the lower limit of  $v_2$  indicated here must be greater than about 54.0 unless we admit there has been an extraordinary loss of protein.

All the evidence, then, is in agreement that the mean change in

<sup>1</sup> Unless the total circulating hemoglobin is actually decreased immediately after the work; this possibility has never been seriously entertained,



blood volume in these experiments was from 6000 cc. in rest to between 5570 and 5600 cc. Within this range, the necessary hemoglobin and serum protein exchanges are presented in Table V.

#### DISCUSSION

The observations reported here are consistent with one another and, qualitatively, consonant only with the interpretation that the major concentration change in the blood resulting from the brief severe work is due to movement of a filtrate low in protein from the blood. Furthermore, the accession of new red cells to the blood must be small but not infinitesimal and there must be either a small loss of protein from the blood serum or a considerable change in its physical properties.

TABLE V  
*Final Limits for Movement of Red Cells and of Serum Protein; Average of Present Experiments*

Final blood volume (1)	Total volume of new red cells (2)	Per cent of total red cells (3)	Total protein lost (4)	Per cent of total protein (5)
cc.	cc.		gm.	
5570	2	0.09	5.5	2.3
5580	8	0.30	4.9	2.1
5590	12	0.45	4.5	1.9
5600	18	0.53	4.1	1.7
5610	22	0.82	3.7	1.6
5620	24	0.90	3.3	1.4

The quantitative analysis developed for the colloid osmotic pressure data may be subjected to certain criticisms, the principal one being that the measured colloid osmotic pressure includes Donnan effects. The Donnan effect arises from the fact that proteins are not electrically neutral. For any part of the change in the gross colloid osmotic pressure to be due to other than changes in the average molecular dimensions of the proteins present, there must be changes in the ionization of the proteins or changes in the ionic constitution of the crystalloid solution phase.

The method for the determination of colloid osmotic pressure is specifically designed to maintain constant both the protein ionization and the ionic constitution of the solvent phase. Most

of the difference in pH between the rest and the work blood samples is eliminated by the suspending buffer solution employed. Likewise, changes in the crystalloid ions must be very nearly completely negated by the large amounts of external phase used here. The fact that, under the conditions of the measurement, both pH and ionic strength, inside and outside the collodion membrane, are closely constant throughout all the measurements makes it unlikely that any appreciable change in the Donnan effect can be involved. It may be pointed out in this connection that various workers (*e.g.*, Marrack and Hewitt, 1927) have shown that the gross colloid osmotic pressure of blood serum is little affected by changes in pH of several tenths and is also very little altered by moderate changes in salt concentration (see Meyer, 1932).

Earlier in this paper comparison was made with the results of Dill, Talbott, and Edwards (1930) which indicated that with more prolonged, less severe work simple fluid movement will account for the observed concentration changes more completely than in the present experiments. This seems reasonable in view of the fact that in our experiments the strain on the circulatory system is much more intense. The difference may well indicate that protein filtration out from the blood stream is normally of small magnitude and that really excessive stress is required to cause more than an insignificant protein leakage.

The indications are, in the present experiments, that the fluid filtered out from the blood stream in this brief exhausting work averages about 1 per cent in protein concentration. White, Field, and Drinker (1933) reported tissue lymph concentrations of from 0.5 to 1.5 per cent in dogs when lymph flow is at a high level. We cannot say, however, that in our experiments the blood filtrate is removed in the lymphatics; some of it undoubtedly is thus returned but the rapidity of readjustment following the work inclines us to the belief that much of the filtrate returns to the blood stream by the same route it left it in work. For example, a typical illustration of the rate of recovery following this brief severe exercise is given in Table VI.

For figures like those in Table VI to be explained by lymph return to the blood would seem to us to require an improbably large lymph flow, even if we accept the larger estimates of lymph flow from Drinker and Field (1933).

The various points of significance of the present work are obvious, but it should be pointed out that, among other things, we find little evidence for a large splenic activity in these short strenuous periods of exercise, in spite of the fact that the indications of reduced blood volume imply marked vasoconstriction in the abdominal cavity during the exercise. This is similar to the findings of Dill, Talbott, and Edwards (1930) for longer, much less severe work in which they found blood concentration changes in splenectomized subjects similar to those in normals.

## SUMMARY

Normal young men were exercised to exhaustion on a treadmill in a period of 1 minute and blood from prolonged rest immediately

TABLE VI

*Recovery Following Running to Exhaustion in 1 Minute*

Subject D. K., football player in training.

Time after work, min. ....	Rest	$\frac{1}{2}$	1	$2\frac{1}{2}$	$3\frac{1}{2}$	11	25	50	92
Blood O <sub>2</sub> capacity, vol. per cent. ....	21.21	23.13	23.41	23.25	22.92	22.48	22.06	21.45	20.90
Plasma organic solids, gm. per 100 cc. ....	7.89	9.24	9.20	9.13	9.10	8.97	8.68	8.05	7.86

before the work was compared with blood drawn within 1 to 2 minutes after the work. Oxygen capacity, serum protein, and serum colloidal osmotic pressure in particular were studied.

In contrast to large increases in oxygen capacity and in spite of even larger (averaging 12.5 per cent) increases in serum protein concentration, the colloidal osmotic pressure generally decreased. Reasons are given for believing that this decrease is directly related to a change of the mean size of the protein molecules.

A method of analysis by successive approximations is presented. From its application to the present data it is indicated that, as an average for our subjects, about 400 cc. of filtrate containing about 1 per cent protein left the blood during work and that at the same time something between 5 and 20 cc. of new red cells entered the active circulation.

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## **CYSTINURIA\***

### **II. THE METABOLISM OF CYSTINE, CYSTEINE, METHIONINE, AND GLUTATHIONE**

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It has been repeatedly demonstrated that the level of cystine excretion by a cystinuric is not increased by the ingestion of this amino acid but is augmented by the feeding of proteins, especially those rich in sulfur. The ability of the cystinuric organism to oxidize free cystine and its apparent failure to utilize this amino acid as it exists in the protein molecule have always been difficult to understand. The inability to formulate a reasonable explanation of this finding pointed to a fundamental gap in our knowledge of intermediary sulfur metabolism. Some light was thrown on the problem by our observation that cystine is possibly excreted in the urine, not as the free amino acid, but as a complex of unknown nature which decomposes with the liberation of free cystine (1). No further insight into the processes possibly involved could be gained from the literature, because, among the naturally occurring sulfur compounds, cystine alone has been studied in cystinuria since the advent of new and specific methods for the quantitative determination of this amino acid.

Recently a new aspect has become apparent with the establishment of a close interrelationship between cystine and methionine. It has been observed (2) that methionine can replace cystine in its stimulating effect on the growth of rats subsisting on diets deficient in these amino acids. Moreover, it has been found (3) that methi-

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onine may function as does cystine in the detoxication of bromobenzene.<sup>1</sup>

Various explanations of these findings have been offered. White and Lewis (3) seem to prefer the suggestion that cystine and methionine have some common product of intermediary metabolism and that methionine may have a cystine-sparing action. The experimental evidence, however, may also indicate a possible conversion of methionine to cystine (*cf.* (2) p. 476). The conversion of methionine into cystine *in vivo* is difficult to formulate chemically. It was thought that this interpretation could be further tested by experiments on a cystinuric individual. Indeed it had been suggested by Jackson and Block (2) that an increased urinary cystine excretion after the ingestion of methionine by a cystinuric "would again be tantamount to proof that the body can convert methionine to cystine."

The metabolism of methionine, therefore, was investigated in a case of cystinuria, and, for purposes of comparison, cystine, cysteine, and glutathione were included in the study (*cf.* (6)). The experiments reported on the following pages indicate that when *cysteine or methionine* is ingested by a cystinuric, only a small portion of these compounds is oxidized, the larger part appearing in the urine as extra *cystine*. Cystine and glutathione ( $-SH$ ), on the other hand, are almost completely oxidized; a small portion of the latter may appear in the urine as extra cystine.

#### EXPERIMENTAL

*Case History*—The patient was a 15 year-old male whose right kidney had been completely destroyed by cystine stones, necessitating its surgical removal by one of us (C.). Shortly after recovery from the operation, the metabolic studies were undertaken (for details *cf.* (5)).

*Diet*—The patient was placed on a weighed, meat-free diet<sup>2</sup> of constant sulfur content (350 gm. of carbohydrate, 100 gm. of fat, and 50 gm. of protein). The daily intake of milk, eggs, bread,

<sup>1</sup> *Cf.* Brand and Harris (4, 5) regarding a possible mechanism for the formation of bromophenylmercapturic acid and the source of its acetyl group.

<sup>2</sup> We are indebted to Miss M. E. Thompson, Therapeutic Dietitian of the Presbyterian Hospital, for her cooperation in these studies.

cheese, and butter was constant but the vegetables, fruits, and cereals were varied. Table I indicates that on account of the close cooperation of the subject, the daily excretion of the nitrogenous constituents as well as of sulfur and of cystine during the control periods was constant within a strikingly narrow range.

### *Methods*

The various urinary constituents determined in these studies are recorded in Table I. The following methods were used: pH, colorimetric; total nitrogen, Kjeldahl; urea, urease (Van Slyke and Cullen); ammonia, aeration (Folin); amino acid nitrogen, colorimetric (Folin); creatine and creatinine, Kassell<sup>3</sup> (9); inorganic phosphorus, colorimetric (Fiske and Subbarow); total sulfur, gravimetric (Benedict and Denis); inorganic and total sulfates, gravimetric (Folin); cystine, Folin and Sullivan.

The Folin determination of cystine was carried out according to Rimington (10); however, the new color reagent (11) containing lithium carbonate and phosphoric acid (12) was employed. It should be kept in mind that owing to interfering substances the results tend to be somewhat high (*cf.* (13)). Other —SS— and —SH compounds besides cystine and cysteine are determined by this procedure.

On the other hand the cystine values indicated by our modification (1) of the specific<sup>4</sup> Sullivan method are about 10 per cent too low. The discrepancy between the two methods must be borne in mind when Table I is studied. In order to obtain satisfactory results with the Sullivan method it is important to use freshly oxidized  $\alpha$ -naphthoquinone-4-sulfonate.<sup>5</sup> Determinations were frequently carried out in triplicate and checked by repetition.

The neutral sulfur is obtained by subtracting the amount of

<sup>3</sup> In order to obtain correct values in the creatinine determination with the Pulfrich photometer, it is necessary to construct suitable calibration tables (we shall be glad to furnish such data upon request). A statement to the contrary by Lieb and Zacherl (7) involves a line of reasoning at variance with the fundamental principles of the instrument, outlined in the handbook issued by the manufacturer (8).

<sup>4</sup> Our observation (1) that cystine methylester gives a more intense Sullivan reaction than cystine itself has been confirmed recently for carboxyl-substituted cystine derivatives (14).

<sup>5</sup> We are indebted to Dr. M. X. Sullivan for this suggestion.



total sulfates from the total sulfur. The neutral sulfur fraction contains, besides cystine, a variety of organic and inorganic sulfur compounds. The latter are designated as "Undetermined neutral sulfur," which is found by deducting the cystine sulfur as determined by the Folin method from the "Total neutral sulfur."

*Metabolic Observations*—After a short time on the basal diet, the daily excretion of the various urinary constituents remained quite constant. Our observations, the majority of which are reported in Table I, were continuous from May 9 to August 5, 1933. The excretion of total nitrogen and of the various sulfur fractions during the control periods dropped somewhat from the beginning (Period 1) to the end of the study (Period 18). For example, the daily excretion of the total nitrogen, sulfur, inorganic sulfate, neutral sulfur, and cystine was 6.1, 0.56, 0.25, 0.27, and 0.86 gm. respectively during Period 1, and 5.3, 0.54, 0.24, 0.25, and 0.77 gm. in control Period 18. This slight decrease in the nitrogen, sulfur, and cystine excretion during the 3 month period was so gradual that it was practically negligible from one feeding period to the next. This slow drop might have been due to a number of factors, such as the institution of an adequate diet after the patient had been on a regimen presumably deficient in protein and cystine for a long time, to the nitrogen- and sulfur-sparing action of the amino acids which were fed, or to variations in the growth processes of the adolescent subject.

Four compounds containing sulfur were fed to the patient during the 3 months in which these metabolic observations were carried out. There was a total of ten periods of 3 days each when the compounds were ingested, preceded and followed by a control period of sufficient duration to insure the return of the subject to a basal condition. Cysteine and cystine were fed three times, while methionine and glutathione (reduced) were given only twice. In order to save space, the results of only two of the cystine and cysteine experiments are presented in Table I. The results of the other experiments which are not given in Table I were in complete accord with those presented.

*Cystine (Periods 4 and 10)*—The results given in Table I show clearly that feeding as much as 6.4 gm. of *l*-cystine did not change the urinary cystine, but that there was a great increase in the excretion of inorganic sulfates and a slight augmentation of unde-

TABLE I  
Metabolic Observations

PERIOD	DATE	SUBSTANCE FED	URINE																	
			pH	NITROGEN					CREATININE		SULFUR				CYSTINE					
				TOTAL	UREA	NH <sub>3</sub>	CREATININE + CREATINOL	AMINO ACID	UNDETERMINED	PREFORMED	TOTAL	INORGANIC P	TOTAL	INORGANIC SO <sub>4</sub>	ETHERAL SO <sub>4</sub>	NEUTRAL	CYSTINE	UNDETERMINED		
																			TOLIN	SULLIVAN
gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm			
10	1933			gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm			
1*	9-11			70	61	45	2	46	12	8	111	134	69	56	25	04	27	25	04	86
2	12	11	66	66	46	2	49	16	11	118	133	71	60	25	06	29	28	01	106	
3*	13	11	68	66	45	2	50	12	13	121	136	74	71	31	03	37	30	07	115	
4	14-15	11	75	58	36	4	42	19	11	108	129	68	53	23	05	27	23	06	85	
5	16	8	68	61	57	2	44	14	16	112	135	76	61	31	05	23	23	02	87	
6	17	0	68	68	45	3	54	19	13	122	145	81	73	40	05	28	23	05	87	
7	18	0	70	58	40	2	44	23	10	103	119	57	62	34	03	28	21	07	78	
8a	19	0	70	73	46	3	52	21	13	118	140	70	61	25	04	30	23	07	85	
9	24		70	65	48	2	51	18	9	121	138	66	57	25	04	28	21	07	80	
10	25	d.c.	70	56	38	2	47	17	10	111	128	68	63	27	03	33	22	11	83	
11	26	METHIO- NINE	1	69	62	40	2	54	27	12	114	146	69	64	25	06	33	27	08	100
12	27	2	68	60	40	2	50	26	10	119	136	60	73	22	06	40	28	12	106	
13	28	2	77	58	36	5	47	36	9	114	126	64	59	23	05	30	27	03	102	
14*	29-JUNE		72	67	42	3	51	26	8	113	138	68	55	23	05	26	22	04	84	
15	30	2	70	60	43	2	49	24	7	109	132	71	62	32	04	26	23	05	86	
16	1	2	71	52	37	2	42	25	6	99	114	59	57	25	05	27	22	05	81	
17	2	4	70	70	47	3	50	26	13	116	135	71	80	45	05	30	26	04	98	
18a	3	4	68	56	39	2	49	22	8	109	133	61	58	26	06	26	22	07	83	
19	4	4	78	59	39	6	50	13	8	114	136	62	59	28	06	25	22	05	84	
20*	7-13		70	59	41	2	50	20	9	111	134	68	55	23	05	26	22	06	88	
21	14	16	68	60	41	3	48	14	8	110	130	61	68	33	06	29	19	10	72	
22	15	16	66	66	42	4	48	12	12	107	130	62	74	31	05	22	19	05	72	
23	16	32	66	66	45	4	50	14	9	107	134	57	74	31	06	32	20	12	74	
24	17	32	68	56	41	3	48	71	7	106	130	63	62	33	05	24	20	04	74	
25	18-21		71	63	44	2	49	13	9	108	132	62	56	26	05	25	20	05	76	
26	22	22	73	62	44	2	52	12	10	114	137	68	67	30	04	33	28	05	103	
27	23	22	68	50	37	2	53	14	12	114	143	67	75	35	05	35	32	03	104	
28	24	22	67	54	35	3	50	25	10	105	136	54	87	30	05	44	40	04	150	
29	25	22	76	48	34	2	50	13	6	96	135	56	39	27	04	33	29	04	108	
30	26	22	76	61	40	4	52	14	9	117	140	76	71	34	07	30	27	05	101	
31*	27	22	70	58	39	2	53	24	10	107	142	75	51	18	06	27	24	03	88	
32*	28-JUNE		70	53	39	2	47	24	6	105	128	60	32	25	05	24	20	04	76	
33	3	2	79	56	34	8	44	23	8	102	120	50	67	24	05	32	24	04	90	
34	4	2	71	58	38	3	49	26	10	110	133	47	74	29	05	40	31	09	115	
35	5	2	71	57	32	3	48	23	15	107	130	46	87	34	04	49	37	12	122	
36	6	2	68	52	33	3	48	26	9	106	129	70	70	27	05	38	35	03	130	
37	7	2	68	48	31	2	50	23	8	106	136	57	65	30	05	30	24	06	91	
38a	8	2	68	75	34	3	54	24	11	131	147	88	77	39	08	30	22	08	70	
39*	9-14		71	53	42	2	48	22	7	107	129	63	56	28	05	25	19	06	77	
40*	15-24		71	53	39	2	45	22	9	108	122	64	52	23	05	25	19	06	77	
41	25	7	73	50	42	2	44	13	7	111	125	60	60	43	05	30	25	07	81	
42	26	7	66	49	39	3	40	13	2	99	108	60	67	36	03	26	22	04	82	
43	27	8	69	57	46	3	48	13	2	120	130	68	110	69	05	36	26	12	89	
44	28	8	69	55	44	2	46	13	2	111	129	68	63	57	04	31	26	07	91	
45	29	8	71	54	44	1	41	11	3	103	110	69	61	28	06	27	21	06	78	
46	30	8	74	59	47	2	43	17	4	109	117	70	62	30	04	32	23	09	87	
47*	31-JULY		73	53	41	2	44	15	4	108	120	64	54	28	05	28	21	07	77	
48	1	4	78	53	39	2	50	11	4	119	133	63	68	28	05	29	20	03	77	
49	2	4	76	52	35	4	48	16	2	123	140	73	73	33	06	34	24	04	108	
50	3	4	70	46	29	3	46	15	2	114	136	54	34	19	05	30	24	04	78	
51	4	4	78	52	34	2	47	14	5	110	127	52	20	04	28	22	06	04	78	

\* IN THESE PERIODS FIGURES INDICATE AVERAGE DAILY EXCRETION

terminated neutral sulfur. These experimental observations are in agreement with the literature.

*Cysteine*<sup>6</sup> (Periods 2, 12, 20)—It will be seen in Table I that the ingestion<sup>7</sup> of cysteine resulted in a definite rise in the cystine and neutral sulfur excretion, while there was only a small increase in the production of inorganic sulfates. It will be noticed that on the last day of Period 12, the cystine excreted in the urine had risen to approximately double that of the control period. Extra cystine continued to appear in the urine for some 3 days after cysteine feeding had been stopped.

Although the administration of almost 9 gm. of cysteine hydrochloride caused a decided rise in the output of urinary cystine and of neutral sulfur, nevertheless *cysteine* was *not* found in the urine at any time, as determined by the failure of the urine to give a nitroprusside test before reduction with sodium cyanide. This was further substantiated by the fact that the cysteine feeding did not change the excretion in the urine of those substances which react with the Folin cystine reagent without reduction with sodium sulfite (*cf.* (13)). The detailed figures for the cystine determination by the Folin method for Periods 11 to 13 are given in Table II

<sup>6</sup> In a case of cystinuria, Gross (15) determined by the Gaskell method (acetone-acetic acid precipitation) the amount of cystine excreted in daily morning specimens. He administered cystine and cysteine to the patient and found that the cystine precipitate in the morning specimen was increased after the ingestion of the latter amino acid only. The finding has occasioned very little comment in the literature, perhaps on account of the apparent inadequacy of the experimental procedure. The effect of the administration of cysteine on the urinary sulfur partition in cystinuria was also investigated by Wolf and Shaffer (16). They concluded that "cystin and cystein, administered by the mouth are completely catabolized to sulfates" (p. 468). The single cysteine feeding experiment reported by them in Table II (p. 451), however, shows that the excretion of neutral sulfur was higher after the administration of 3 gm. of cysteine than after the ingestion, in the preceding experiment, of the same amount of cystine. Whether or not a rise in neutral sulfur in these experiments was due to extra cystine could not be ascertained without the use of methods, developed since Wolf and Shaffer's important contribution, for the direct determination of this amino acid. Their findings, therefore, are not necessarily in contradiction to ours, in spite of their interpretation of their experiments.

<sup>7</sup> Cysteine hydrochloride was dissolved in water, neutralized with sodium bicarbonate, and immediately ingested by the patient.

in which the results are shown with and without sulfite respectively, while the difference between these values (indicating cystine) is also recorded.

The increase in cystine excretion in the cysteine experiments in Periods 12 and 20 was observed both with the Folin and the Sullivan methods. The discrepancy in the cystine figures obtained with these two methods was discussed above.

*Methionine (Periods 6 and 14)*—The ingestion of *dl*-methionine by the cystinuric resulted in only a small increase in the output of inorganic sulfates and of undetermined neutral sulfur (presumably

TABLE II  
*Details of Folin Determination for Cystine in Cysteine Experiment*

Period No.	Date	Substance fed		With sulfite, calculated as cystine (a)	Without sulfite, calculated as cystine (b)	Cystine (a - b)
	1933		gm.	gm.	gm.	gm.
11	June 18	Cysteine HCl		1.29	0.54	0.75
	" 19			1.26	0.51	0.75
	" 20			1.30	0.51	0.79
	" 21			1.25	0.51	0.74
12	" 22		2.2	1.58	0.55	1.03
	" 23		2.2	1.69	0.50	1.19
	" 24		4.4	1.97	0.47	1.50
12-a	" 25			1.55	0.47	1.08
	" 26			1.54	0.53	1.01
	" 27			1.41	0.53	0.88
13	" 28			1.28	0.52	0.76
	" 29			1.27	0.51	0.76

unchanged *d*-methionine), but a great increase in the excretion of total neutral sulfur and of cystine was observed both with the Folin and the Sullivan methods. In this case, as in the cysteine experiment, the appearance of extra cystine in the urine continued for 3 days after the last dose of methionine, and also the level of cystine excretion was almost doubled at times. It was again shown by means of the nitroprusside and Folin reactions that only extra cystine and no cysteine was present in the urine after methionine administration (detailed figures similar to those given in Table II are omitted).

*Glutathione*<sup>8</sup> (Periods 8 and 17)—The feeding of 8 and of 16 gm. of glutathione (—SH) resulted in only a very slight increment in the cystine and undetermined neutral sulfur excretion. The largest part of the peptide was oxidized to sulfuric acid.

Further analyses of the data are presented in Tables III and IV and in Fig. 1. For purposes of comparison, the average daily excretion of the various urinary sulfur constituents was calculated

TABLE III

*Average Daily Urinary Sulfur Partition in Control and Experimental Periods*

Period No.	Substance fed	Per cent total S			
		Inorganic sulfate S	Neutral S	Cystine S	Undetermined neutral S
		per cent	per cent	per cent	per cent
1		45	47	41	7
9		45	45	40	6
18		44	46	39	7
10	Cystine	66	28	19	8
17	Glutathione	58	36	27	9
12	Cysteine	45	49	43	5
14	Methionine	39	54	42	13

TABLE IV

*Extra Sulfur and Extra Cystine in Experimental Periods*

Period No.	Substance fed		Amount of extra S			Extra cystine excreted
			Fed	Excreted		
		gm.	gm.	gm.	per cent	gm.
10-10-a	Cystine	6.4	1.71	1.36	80	None
17-17-a	Glutathione	16.0	1.63	1.28	79	0.59
12-12-a	Cysteine HCl	8.8	1.78	0.86	48	2.13
14-14-a	dl-Methionine	8.0	1.72	1.10	64	2.03

for certain periods and the percentage distribution of the sulfur partition established (Table III). It can be seen that the sulfur partition is constant for the three control periods (Periods 1, 9, 18). The typical cystinuric distribution of the sulfur partition is con-

<sup>8</sup> "It has been shown in some unpublished work by Hele, Hopkins, Lawrie, Leese, Meldrum and Pirie that a cystinuric could oxidise 3 g. of glutathione, given by mouth, as readily as cystine" (17).

siderably altered towards the normal in the cystine and glutathione periods (Periods 10 and 17) in which there is a marked increase in the proportion of inorganic sulfate and a corresponding decrease in the percentage of cystine sulfur. On the other hand, in the cysteine and methionine feeding periods (Periods 12 and 14) the characteristic sulfur partition is strictly maintained. Table III, thus, shows that the essential metabolic error in cystinuria is concerned with cysteine and methionine.

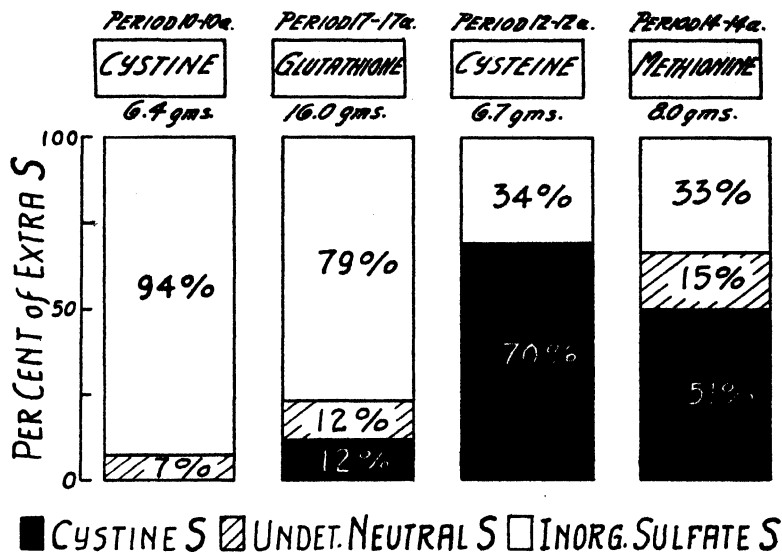


FIG. 1. Partition of extra sulfur in experimental periods. The amounts of the substances fed are given in gm.

A summary of the data on the extra sulfur excreted in the feeding periods is given in Table IV. The extra sulfur is calculated as in the following example.

Total S, Period 10-10-a (4 days)	= 3.58 gm. minus
" " daily average, Periods 9 and 11, $\times 4$	= 2.22 "
Extra total S, Period 10-10-a	= 1.36 gm.

The other data reported in Table IV and Fig. 1 were similarly determined. The figures for extra sulfur are, however, somewhat

low, because the ingestion of these sulfur compounds produces some nitrogen- and sulfur-sparing action, resulting in a fall in the basal level. Nevertheless, this method, with carefully conducted fore, after, and adjustment periods appears to be superior to other methods of calculation.

It can be seen from Table IV and Fig. 1 that about 80 per cent of the ingested cystine and glutathione sulfur can be accounted for as extra sulfur, while in the case of cysteine and methionine only 48 and 64 per cent respectively were found. Cystine and glutathione are almost completely oxidized to inorganic sulfate, while only approximately one-third of the cysteine and methionine is so converted. After cystine feeding there is no extra cystine, while with glutathione, cysteine, and methionine,<sup>9</sup> respectively, 0.59, 2.13, and 2.03 gm. of extra cystine were found, corresponding to 12, 70, and 51 per cent of the extra sulfur.

Table I shows that the extra sulfur derived from cystine and glutathione appears in the urine much more rapidly than that from cysteine and methionine. The implications of this observation on the nitrogen to sulfur ratio as well as an analysis of the other data presented in Table I will be given in a subsequent communication on the effect of the ingestion of proteins by the cystinuric.

The experiments with cystine, cysteine, and methionine were repeated on a second case of cystinuria with essentially the same results (*cf.* (18)).

Our studies on the metabolic behavior of the sulfur compounds mentioned above in normal human beings and patients with muscular dystrophy will be the subject of a separate communication.

#### DISCUSSION

These studies tend to show that the metabolism of cystine may be quite different (Fig. 1) from that of cysteine<sup>10</sup> and that these two compounds are not as interchangeable in intermediary metabolism

<sup>9</sup> Part of the extra sulfur after the feeding of *dl*-methionine is presumably unchanged amino acid. If the values in Table IV were corrected accordingly, then the sulfur partition of the methionine and cysteine experiments would be almost the same.

<sup>10</sup> This also holds true for homocystine (18-20) and homocysteine (unpublished data).

as has been assumed generally (*cf.* (21, 22)). On the contrary, we must think of cystine and cysteine as two individual and separate amino acids. Even if we accept physicochemical experiments on pure solutions of cystine and cysteine to indicate that this system satisfies the requirements for thermodynamic reversibility (23), still the studies on the cystinuric show that the oxidation of cysteine to cystine takes place with greater ease than the reverse reaction. However, other experiments demonstrate that under conditions, particularly under stress (*e.g.* sulfur-deficient diets, bromobenzene feeding, certain *in vitro* experiments (24), etc.), the animal organism can probably reduce cystine and certain derivatives thereof (25).

The experiments carried out on the living organism indicate that there are probably separate mechanisms for the oxidation of —SS— and —SH compounds. A mechanism for the oxidation of —SH compounds was recently demonstrated in kidney and also in liver by the *in vitro* experiments of Pirie (24) who failed, under his conditions, to find any evidence for the direct oxidation of cystine. The experiments presented in this paper show that cystine can be oxidized without previous reduction to cysteine.

We do not believe that our findings can be explained by a differential rate of absorption<sup>11</sup> for cystine and cysteine (27). In this connection reference should be made to Table I which clearly shows that the latter amino acid, notwithstanding its somewhat more rapid absorption, is much more slowly metabolized (*cf.* (28)).

The observation that only extra cystine and no cysteine is found in the 24 hour specimens of urine following the administration of the latter amino acid indicates the possible rôle of the kidney in the oxidation-reduction mechanism of the —SS— $\leftrightarrow$ —SH system. The earlier studies (1) suggest that cystine, at times, is not excreted by the cystinuric as the free amino acid but as some labile compound (a thio-substituted cysteine?) which spontaneously decomposes in the urine to yield cystine. The nature and significance of this phenomenon and its possible bearing on stone formation in cystinuria remain obscure.

It has been proposed (24) that the first step in the oxidation of

<sup>11</sup> The possible significance of experiments by London *et al.* (26) on the deamination of alanine and aspartic acid by the intestinal wall will be discussed in a subsequent communication.



glutathione is the hydrolysis of the tripeptide into its constituent amino acids. If this were so, then the ingestion of glutathione should liberate considerable quantities of free cysteine. This should result in a great rise in the extra cystine as shown by our cysteine feeding experiments. However, the administration of reduced glutathione to our patient resulted in only a very slight rise in urinary cystine excretion, the largest part of the glutathione being oxidized to inorganic sulfate (Fig. 1). Therefore, the primary hydrolysis of glutathione( $\text{—SH}$ ) is probably only one of the pathways, perhaps of minor importance, by which it can be catabolized. The greater part is either first oxidized to  $\text{—SS—}$  glutathione or both the  $\text{—SS—}$  and  $\text{—SH}$  forms are metabolized in an unconventional way, perhaps along the lines suggested by us in a previous communication (4, 5). Thus the metabolic behavior of an amino acid may vary markedly, depending upon whether it is catabolized as the free amino acid or in combined form as a peptide.

It may be of interest to consider the results of the feeding experiments with cysteine, glutathione, and cystine in the light of our knowledge of their behavior during alkaline degradation (29–31). Thus, it has been found that the cysteine molecule as a whole is distinctly more resistant towards alkaline reagents than are cystine and glutathione. These *in vitro* observations may have some relation<sup>12</sup> to the finding that the latter substances are oxidized by the cystinuric more completely and more rapidly than is cysteine.

Regarding the metabolic interrelationship between cystine and methionine (2, 3), our findings point to a conversion of methionine into *cysteine*. Little is known of other pathways in the catabolism of methionine (for glucose formation *cf.* (32)) and even its conversion into cysteine is still obscure. However, unpublished experiments show that the initial reaction is probably a demethylation of methionine to homocysteine, since the latter amino acid fed to a cystinuric produces a great rise in cystine excretion (*cf.* (18)).

The experiments on the ingestion of sulfur amino acids indicate that the fundamental difficulty in the cystinuric individual arises not from an inability to handle the cystine but rather the methionine in the food and also the cysteine which may be formed during protein digestion. This view finds further support in recently completed experiments on the feeding of casein and lactalbumin to

<sup>12</sup> We are indebted to Professor H. T. Clarke for this suggestion.

a cystinuric subject. Although the cystine excretion in cystinuria is caused mainly by dietary methionine, the inborn error of metabolism is concerned with the handling of cysteine. It is possible that the kidney is the site of this metabolic disturbance.

The metabolic origin of the sulfhydryl group in biologically important compounds is not clear as yet, but it seems that, under conditions, methionine rather than cystine is its chief source. In view of the apparent ease with which  $-\text{SH}$  is converted into  $-\text{SS}-$ , both in biological as well as in purely chemical systems, it appears that the potential  $-\text{SH}$  group in methionine is protected by a methyl group and is liberated by a process of demethylation. It should be considered that the methyl radical liberated from methionine is not necessarily oxidized but that it may be transferred to other compounds; *e.g.*, to furnish the methyl group in creatine.<sup>13</sup>

It should be further pointed out, in the light of our experiments, that the large amounts of cystine found in skin, nails, and hair may be derived, to a considerable extent, from cysteine and dietary methionine rather than from cystine itself.

For many years cystine has been thought an essential amino acid; *i.e.*, an absolutely indispensable dietary component which cannot be synthesized by the animal organism. The experiments of Jackson and Block (2) and of White and Lewis (3), together with the findings presented in this paper, make this statement no longer entirely true, since cystine can apparently be synthesized from methionine.

#### SUMMARY

1. The metabolism of cystine, cysteine, methionine, and glutathione was investigated in a case of cystinuria.

2. Cystine and glutathione are almost completely oxidized, the latter yielding a small amount of extra cystine.

3. Cysteine and methionine are excreted largely as extra cystine, only a small part being oxidized to inorganic sulfate. Only cystine and no cysteine was found in the urine in these experiments.

<sup>13</sup> Chemical and metabolic studies with isocreatine (N-methyl-N'-carboxymethylguanidine) have yielded no evidence in support of a previous suggestion regarding the possible origin of the methyl group of creatine (33).

4. Cystine and cysteine should be considered as two individual and separate amino acids.

5. Cystine can be catabolized without previous reduction to cysteine and glutathione without previous hydrolysis.

6. There are probably separate mechanisms for the oxidation of —SS— and —SH compounds.

7. The metabolic behavior of an amino acid may vary markedly, depending upon whether it is catabolized as the free amino acid or in combined form as a peptide.

8. One of the pathways of methionine catabolism is its conversion into *cysteine*, the first step being probably a demethylation to *homocysteine*.

9. The cystine excretion in cystinuria is caused mainly by dietary methionine, but the inborn error of metabolism is concerned with the handling of *cysteine*.

10. The evidence presented in this paper, together with that from other sources, seems to indicate that cystine is not an essential amino acid, since it can be synthesized from methionine.

11. Further implications of the findings have been discussed.

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## THE EFFECT OF INSULIN ON THE PURINE METABOLISM OF THE DALMATIAN COACH-DOG\*

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In 1916 Benedict (1) discovered that the Dalmatian coach-dog occupies a unique position with regard to its purine metabolism. Not only does this breed differ from others by excreting several times as much endogenous uric acid per kilo of body weight as does man, but it differs also from man and the chimpanzee in that it eliminates a high percentage of allantoin. This metabolic anomaly is true only of the pure bred Dalmatian, for Onslow (2) found that hybrids obtained from a cross between a Dalmatian and a terrier excreted uric acid and allantoin in a proportion similar to that of other breeds.

The Dalmatian, combining in one animal the excretion of both uric acid and allantoin in measurable amounts, provides a fertile field for the study of the metabolism of these two substances. In a previous communication (3) it was shown that insulin increased the excretion of allantoin in the urine of the ordinary breed of dog. It seemed of interest therefore to study the action of this hormone on the purine metabolism of the Dalmatian.

### EXPERIMENTAL

*Care of Animals*—Pure bred adult Dalmatian coach bitches were used. The present investigation deals with two phases of the action of insulin on the purine metabolism of this breed. In the first part, in which the effect of insulin on the excretion of allantoin and uric acid in the urine was studied, the dietary treatment of the animals was similar to that of the normal dogs pre-

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viously reported (3). The animals were maintained on a synthetic Cowgill diet (4) for long periods of time and the hormone injected only after a suitable constancy was observed in the daily amounts of total nitrogen, uric acid, and allantoin excreted in the urine. As reported in the previous communication (3), the dogs were fed once per day and the time intervals of catheterization planned with respect to the time of feeding so that samples of urine were obtained for a 5 hour period prior to feeding and for a 19 hour interval after the ingestion of food.

In the experiments dealing with uric acid of the blood, all the Dalmatians, with the exception of Dog L-A, received a Cowgill diet for several days before the injection of insulin. Dog L-A was maintained on a mixture of meat, fish, cod liver oil, and rice bran. The first sample of blood was removed from these animals as a rule 16 hours after the last meal. 10 units of insulin were then injected and blood samples taken for uric acid and for sugar estimations at stated intervals during the following 7 or 8 hours. The same procedure was adopted in the case of the control experiments except that 0.8 per cent saline was injected instead of insulin.

#### *Analytical Procedures*

Allantoin in the urine was determined by the method described by Read and Chaikoff (5); uric acid in the urine by the direct method of Benedict and Franke (6) with the modifications of Christman and Ravitch (7). For blood uric acid, the recent colorimetric procedure of Folin (8) was employed, with the use of unclaked blood (9). Despite the fact that the Dalmatian excretes several times as much uric acid per kilo of body weight as does man, its blood uric acid is but slightly higher than that of other breeds of dogs. It was therefore found necessary to employ uric acid standards of greater dilution than the one recommended by Folin (8). A series of standards of varying dilutions was prepared for each analysis, and a standard corresponding closely with the unknown was used in the final colorimetric comparison. Blood sugar was determined by the copper-iodometric reagent of Shaffer and Somogyi (10), the filtrate being obtained by the precipitation of blood with zinc hydroxide (11).

### Results

#### *Effect of Insulin on Excretion of Allantoin and Uric Acid in Urine of the Dalmatian Coach-Dog*

*Dog P (Table I)*—This animal received 10 units of insulin at 7.45 a.m. (February 25), and by 12.30 p.m. the excretion of uric acid nitrogen rose to 93 mg. as compared with 42 mg. eliminated during the same interval on the previous day. This effect of insulin must have persisted for some time after the 5 hour interval, for the urine of the next 19 hour period contained an increased amount of uric acid. During the 5 hour interval of the following day, however, the amount of uric acid appearing in the urine was

When the injection of insulin was repeated at the same time 3 days later, an increased elimination of uric acid was again observed, but on this occasion the effect was noted only in the 5 hour interval following the administration of the hormone. A decreased excretion, however, was again found in the 5 hour period of the following day.

The amount of allantoin excreted by Dog P remained constant throughout the whole period of observation despite the injection of insulin on two occasions.

It is important to note that an increased volume of urine was associated with the increased excretion of uric acid. Thus, when the amount of uric acid nitrogen for the 5 hour interval rose from a normal value of 42 mg. to 92 mg. after the injection of insulin (February 25), the volume of urine containing the latter amount was 240 cc. as compared with 125 cc. in which the 42 mg. were eliminated. Again on February 28, when the excretion of uric acid nitrogen rose from 36 to 72 mg., the urine volume for the respective periods of observation increased from 85 to 160 cc.

*Dog D-S (Table I)*—The injection of 10 units of insulin in this animal led to an increase of over 100 per cent in the amount of uric acid excreted during the 5 hours immediately following the administration of the hormone. This effect was accompanied—as had been previously observed in Dog P—by a marked diuresis, the volume of urine for this period increasing by 100 cc. A slight rise in the output of uric acid was also noted in the next 19 hour



TABLE I  
Effect of Insulin on Excretion of Allantoin and Uric Acid by the Dalmatian Dog

	Date	Ammonia N						Urea N						Allantoin N						Uric acid N						Total N						Rest N 24 hrs.	Urine vol- ume 5 hrs.	kg.
		19 hrs.		5 hrs.		24 hrs.		19 hrs.		5 hrs.		24 hrs.		19 hrs.		5 hrs.		24 hrs.		19 hrs.		5 hrs.		24 hrs.		gm.	gm.	gm.						
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.				gm.	gm.				
Dog P	Feb. 22	0.737	0.135	0.872	7.98	0.98	8.96	0.072	0.018	0.090	0.147	0.038	0.185	9.40	1.27	10.67	0.57	100	18.6															
	" 23	0.756	0.141	0.897	8.01	0.90	8.91	0.068	0.018	0.086	0.154	0.039	0.193	9.49	1.20	10.69	0.60	115																
	" 24	0.741	0.134	0.875	8.00	0.97	8.97	0.068	0.019	0.087	0.150	0.042	0.192	9.42	1.27	10.69	0.56	125	18.6															
	" 25	0.744	0.143	0.887	7.94	1.39	9.33	0.069	0.020	0.089	0.138	0.093	0.231	9.35	1.81	11.16	0.62	240																
	" 26	0.736	0.138	0.874	7.64	0.95	8.59	0.073	0.016	0.089	0.168	0.013	0.181	9.14	1.24	10.38	0.65	90	18.6															
	" 27	0.732	0.143	0.875	7.88	0.97	8.85	0.071	0.017	0.088	0.138	0.036	0.174	9.31	1.25	10.56	0.58	85																
	" 28	0.742	0.146	0.888	8.00	1.55	9.55	0.073	0.018	0.091	0.150	0.072	0.222	9.41	1.92	11.33	0.58	160	18.6															
Dog D-S	Mar. 1	0.751	0.140	0.891	7.55	0.87	8.42	0.070	0.019	0.089	0.141	0.020	0.161	9.02	1.18	10.20	0.64	95	18.6															
	" 2	0.801			7.97		0.068				0.148			9.48																				
	" 3	0.801	0.138	0.939	6.73	1.05	7.78	0.070	0.019	0.089	0.112	0.029	0.141	8.20	1.32	9.52	0.57	120	20.5															
	" 4	0.740	0.141	0.881	6.86	1.07	7.93	0.065	0.021	0.086	0.108	0.030	0.138	8.32	1.38	9.70	0.66	125																
	" 5	0.783	0.135	0.918	6.69	0.95	7.74	0.063	0.022	0.085	0.107	0.028	0.135	8.21	1.26	9.47	0.59	120	20.5															
	" 6	0.807	0.150	0.957	6.67	1.54	8.21	0.072	0.027	0.099	0.112	0.062	0.174	8.27	1.86	10.13	0.69	220																
	" 7	0.834	0.142	0.976	6.19	0.92	7.11	0.083	0.024	0.107	0.126	0.028	0.154	7.69	1.19	8.88	0.53	110	20.6															
	"	0.812	0.138	0.950	6.84	0.98	7.82	0.075	0.019	0.094	0.102	0.027	0.129	8.35	1.28	9.63	0.64	140																

*Dog P*—This dog was catheterized at 7.30 a.m. and 12.30 p.m. daily; fed at 12.45 p.m. daily. Diet: 210 gm. of diet mixture + 38 gm. of lard + 19 gm. of unsalted butter. Diet N = 5.88 gm. per 100 gm. of diet mixture. On February 25 and 28, 10 units of insulin were injected subcutaneously at 7.45 a.m. The period of 19 hours represents the interval from 12.30 p.m. to 7.30 a.m.; the period of 5 hours, from 7.30 a.m. to 12.30 p.m.

*Dog D-S*—This dog was catheterized at 7.40 a.m. and 12.40 p.m. daily; fed at 12.45 p.m. daily. Diet: 180 gm. of diet mixture + 32 gm. of lard + 16 gm. of unsalted butter. Diet N = 5.85 gm. per 100 gm. of diet mixture. On February 5, 10 units of insulin were injected subcutaneously at 7.45 a.m. The period of 19 hours represents the interval from 12.40 p.m. to 7.40 a.m.; the period of 5 hours, from 7.40 a.m. to 12.40 p.m.

interval. This dog, however, in contrast to Dog P, showed no decrease in the elimination of uric acid in the 5 hour period of the following day. 10 units of insulin failed to influence the amount of allantoin excreted in its urine.

*Effect of Insulin on Blood Uric Acid in the Dalmatian Coach-Dog*

The changes produced by insulin in the uric acid of the blood of four Dalmatian dogs are shown in Table II for Dogs P, D-S, and L-A, and in Chart I for Dog S-S. The subcutaneous injection

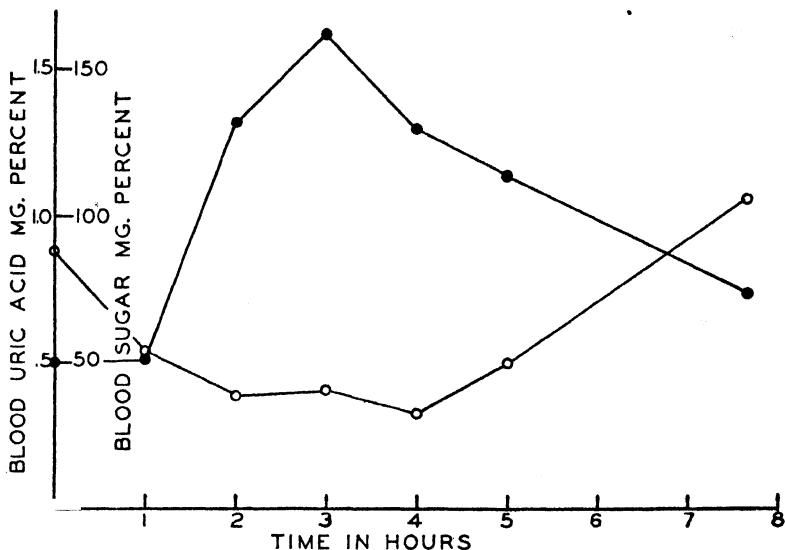


CHART I. The effect of insulin on the blood uric acid and blood sugar of Dalmatian Dog S-S. At 0 hour 10 units of insulin were injected subcutaneously. ● indicates blood uric acid; ○, blood sugar.

of insulin led to an enormous increase in the uric acid content of the blood, the most marked rise occurring in Dog S-S, in which the blood uric acid rose from a normal value of 0.50 mg. per cent to 1.62 mg. per 100 cc. of blood, an increase of over 200 per cent. Maximum values of 1.28, 1.30, and 1.30 mg., which in all animals were observed approximately 3 hours after the injection of the hormone, were obtained in Dogs D-S, P, and L-A respectively, as compared with values of 0.50, 0.44, and 0.44 mg. per cent found

in these animals just prior to the administration of the hormone. It is interesting to note that the blood uric acid in all four animals had not changed 1 hour after the administration of insulin (at which time, however, the blood sugar had already fallen considerably), but that at the 2nd hour following the injection of the hormone the uric acid content of the blood had already risen to

TABLE II  
*Effect of Insulin on Blood Uric Acid of Dalmatian Coach-Dogs*

	Weight	Time	Blood uric acid	Blood sugar
	<i>kg.</i>	<i>min.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Dog P	19.0	0	0.50	80
		2	10 units insulin subcutaneously	
		60	0.52	45
		123	0.78	28
		181	1.28	23
		238	1.14	27
		346	0.82	62
		455	0.44	80
Dog D-S	20.5	0	0.44	79
		2	10 units insulin subcutaneously	
		57	0.44	49
		120	1.13	42
		180	1.30	41
		245	1.16	45
		302	0.99	52
		465	0.57	105
Dog L-A	18.0	0	0.44	83
		3	10 units insulin subcutaneously	
		55	0.48	49
		107	0.87	38
		170	1.30	40
		227	1.14	37
		316	0.94	51
		437	0.56	88

high levels. The return of the blood uric acid to the normal level in these animals was observed between 7 and 8 hours after the injection, the time of this occurrence corresponding closely with the return of the blood sugar to values that were either normal or slightly above normal.

As control experiments, three of the Dalmatian dogs received subcutaneous injections of 2 cc. of sterile 0.8 per cent saline. No

significant changes were found in the uric acid content of the blood during the 7 or 8 hours following the injection of the saline.

*Effect of Insulin on Blood Uric Acid of the Dalmatian Coach-Dog Ingesting Glucose*

Three animals received 100 gm. of glucose by mouth and 30 minutes thereafter 10 units of insulin (Chart II). In one of these dogs, namely Dog L-A, the blood sugar remained at or above the normal

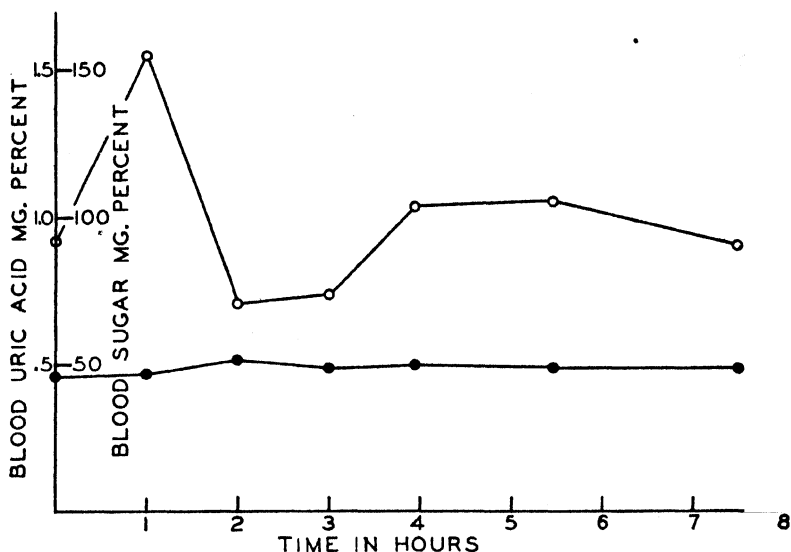


CHART II. The effect of glucose along with insulin on blood uric acid and blood sugar of Dalmatian Dog D-S. At 15 minutes 100 gm. of glucose were fed; at 45 minutes 10 units of insulin were injected subcutaneously. ● indicates blood uric acid; ○, blood sugar.

level throughout the whole period of observation, whereas in Dogs P and D-S, although the blood sugar rose as high as 155 mg. per cent during the course of the experiments, it nevertheless dropped below the normal level for short intervals. The blood uric acid in all three animals showed no significant alterations. Thus the initial values prior to the administration of insulin and glucose were 0.43, 0.46, and 0.46 mg. per cent in Dogs L-A, P, and D-S respectively, and the maximum values observed during the course

of these experiments were respectively 0.55, 0.54, and 0.52 mg. per cent. These results show that insulin does not influence the purine metabolism of the Dalmatian dog in the absence of hypoglycemia.

#### DISCUSSION

The ability of insulin to influence purine metabolism has been demonstrated both in the dog of ordinary breed (3) and in the Dalmatian. In the former, in which allantoin is the chief end-product of purine metabolism, a pronounced increase in the excretion of this substance in the urine followed the subcutaneous administration of insulin. The Dalmatian responded to the injection of insulin by a considerable increase in the elimination of uric acid in the urine, the amount of allantoin in the urine, curiously enough, remaining unchanged despite the fact that an increased urinary volume was observed during the period in which the greater amount of uric acid made its appearance.

The relation of the kidneys to the increased elimination of uric acid by the Dalmatian coach-dog is obviously of significance in any interpretation to be placed upon this effect of insulin. That a diuresis can bring about an increased excretion of uric acid in the urine has been shown by Krafka (12) for the Dalmatian coach-dog. It is therefore of importance to know whether insulin caused an increased excretion of uric acid by means of a diuresis or whether the diuresis was secondary to a piling up of uric acid in the blood and kidneys. Suggestive evidence that this effect of insulin on the Dalmatian is not entirely the result of a diuresis is offered by the experiments on the dog of ordinary breed in which an increased elimination of allantoin in the absence of a change in urinary volume was observed after the injection of insulin. Moreover, although it is conceivable that once the diuresis has been established some "washing-out" of uric acid from the kidneys may have occurred, nevertheless the accumulation of uric acid in the blood of the Dalmatian observed simultaneously with the increased elimination in the urine definitely places the main seat of action of insulin upon purine metabolism elsewhere than in the kidneys. Indeed, the parallelism in the rise of uric acid in blood and urine leaves little doubt that uric acid of the blood is the source of

some, if not all, of the extra uric acid that appeared in the urine of the Dalmatian coach-dog following the administration of insulin.

The difference in the response of allantoin to insulin in the two breeds of dogs is striking. In the Dalmatians 10 units of the hormone produced no change whatsoever in the elimination of allantoin, whereas in the ordinary breed the injection of an equivalent dose of insulin per kilo of body weight practically doubled the excretion of allantoin during the next 5 hours. The interpretation of this variation is beset with difficulties inasmuch as the interrelation of uric acid and allantoin in the Dalmatian dog is as yet poorly understood. It seems definitely established that in the ordinary breed of dog allantoin is the end stage of purine metabolism (13, 14). The results of the present investigation, however, raise the question whether uric acid occupies the same intermediary position in the purine metabolism of both breeds of dogs. That uric acid may be pursuing different paths in the two types of animals has been previously suggested by the observation that most, if not all, of the uric acid administered to the Dalmatian may be quantitatively recovered in the urine (1, 15).

Although the blood sugar of the Dalmatian showed a prompt response to 10 units of insulin, being markedly reduced at the end of the 1st hour after the injection of the hormone, the rise in the blood uric acid was much delayed. Thus at the end of the 1st hour practically no alteration in the level of the blood uric acid was found, despite the fact that the blood sugar had already fallen to values as low as 45 mg. per cent. At the end of the 2nd and 3rd hours, however, the blood uric acid had risen to values several times those of the control period. It would seem that a definite interval, at least 1 hour, exists between the injection of 10 units of insulin and the beginning of the rise in the blood uric acid. The fact that the blood sugar had already fallen before the uric acid began to rise suggested that the increased concentration of uric acid that follows the injection of insulin may be dependent upon the hypoglycemia rather than upon the insulin *per se*. This indeed was shown to be the case by the experiments in which glucose was administered just before the injection of insulin with the

result that no fall in blood sugar occurred throughout the period of observation. Under these conditions no change whatever was obtained in the blood uric acid, although the animal had received 10 units of insulin. These results make it clear, therefore, that hypoglycemia is a necessary precursor to the rise in blood uric acid observed after the injection of this hormone. It does not necessarily follow, however, that under normal conditions the level of the blood uric acid is determined by that of the blood sugar, since the rise in the uric acid of the blood in the present investigation occurred only after the blood sugar had been maintained at lowered levels for at least 1 hour, a condition that seems not to be found normally.

It is interesting to recall in this connection that Quick (16) failed to observe a rise in the blood uric acid of man following the injection of insulin. This may have been due to the absence of hypoglycemia, since sucrose was ingested simultaneously with the administration of insulin. On the other hand, the possibility of a species difference must be recognized in connection with purine metabolism.

#### SUMMARY

A study was made of the effects of insulin on the purine metabolism of the Dalmatian coach-dog.

1. Insulin led to an increased output of uric acid in the urine. The excretion of allantoin was not influenced by the hormone.

2. Insulin produced a marked rise in the uric acid of the blood, the maximum effect being observed 3 hours after the administration of 10 units of the hormone. A peculiarity of this action of insulin was the delay in the beginning of the rise of the blood uric acid. A definite interval of at least 1 hour existed between the time of injection of the hormone and the beginning of this rise. 1 hour after the injection of insulin the uric acid of the blood was still normal despite the fact that the blood sugar had already fallen to low levels.

3. The hyperuricacidemia, which followed the injection of insulin, was dependent upon a hypoglycemia, for when the occurrence of the latter was inhibited by the ingestion of glucose just prior to the administration of the hormone the blood uric acid failed to rise above normal.

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# THE PREPARATION OF THE OPTICALLY ACTIVE ISOMERS OF HOMOCYSTINE AND THE DEMONSTRATION OF THEIR CONFIGURATIONAL RELATIONSHIP TO NATURALLY OCCURRING METHIONINE

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The present study was undertaken in order to make available for physiological studies the optically active isomers of homocystine. The resolution of homocystine was therefore attempted by means of the alkaloid salts of its various acyl derivatives. Much difficulty, however, was encountered. It was suspected that the presence of the meso isomer in the inactive material was very likely the cause of the difficulty. Since no efficient means of separating the meso and racemic isomers from each other has as yet been attained, a new method of approach was desirable whereby interference by the meso form could be avoided.

It is quite apparent that by reduction of homocystine only the racemic form of homocysteine is obtained, no matter which inactive isomer is started with, the meso or racemic. By resolving the reduced material, therefore, the possibility of interference by the meso form could thus be eliminated. However, some means of covering the sulfhydryl group is necessary because the resolution of the free sulfhydryl form obviously presents certain difficulties. For this purpose the sulfhydryl group was covered by a benzyl radical and the resulting *dl*-S-benzylhomocysteine was resolved. This was accomplished by means of the brucine salt of the formyl derivative.

The benzyl derivative was purposely selected on the basis of previous experiences in this laboratory with S-benzyl-L-cysteine (1). It was found that the latter could be converted readily to cystine by reduction in liquid ammonia with metallic sodium fol-

lowed by oxidation of the sodium cysteinate. Furthermore, no detectable racemization was encountered. A similar reduction of *dl*-S-benzylhomocystine followed by oxidation led to excellent yields of homocystine. This method was therefore employed for the conversion of the active isomers of S-benzylhomocystine obtained from the resolution of the N-formyl-*dl*-S-benzylhomocystine to the corresponding active homocystines. Both isomers were obtained in a high degree of purity and in excellent yields.

In order to test the completeness of resolution of the homocystine and, furthermore, to demonstrate the configurational relationship between the active isomers of homocystine and those of methionine, the former were converted to the corresponding methionines by reduction and subsequent methylation. From the homocystine that possessed a rotation of  $[\alpha]_D^{26} = +77^\circ$  in acid solution, *l*-methionine identical with the naturally occurring methionine was obtained, demonstrating that this isomer of homocystine corresponds in spatial configuration to the naturally occurring series of amino acids. Furthermore, the rotation of the methionine so obtained was as high as that isolated by du Vigneaud and Meyer (2) from the enzymatic hydrolysis of casein, thus affording evidence of the completeness of the homocystine resolution presented. The levorotatory homocystine was likewise reduced and methylated, and *d*-methionine was obtained with a rotation as high as that obtained by Windus and Marvel (3) in their resolution of methionine.

Naturally occurring *l*-methionine was also converted to homocystine by refluxing it with 4 volumes of 18 *N* H<sub>2</sub>SO<sub>4</sub> (4, 5) and neutralizing the reaction mixture with NH<sub>4</sub>OH. The homocystine so obtained had a specific rotation of  $+60^\circ$  in HCl. Some racemization had occurred in the reaction as would be expected. The direction of rotation of the product, however, confirmed the configurational relationship found above in the conversion of the homocystine isomers to their corresponding methionines. The formation of this isomer of homocystine from natural methionine by H<sub>2</sub>SO<sub>4</sub> also offers an alternative method for its preparation, for we have found that a sample with such a rotation as that given above can be raised to a maximum by a few recrystallizations from water.

The specific rotations of the above isomers of homocystine were

determined under the conditions usually employed for *l*-cystine, namely a 1 per cent solution of the compound in 1 *N* HCl. However, when the solvent for rotation was changed from HCl to water, the direction of rotation was reversed, similar to the behavior of methionine, but in contrast to that of cystine. It has been shown, for example, that cystine has a negative rotation in water as well as in HCl (6). On the other hand *l*-methionine, which has a negative rotation in water, possesses a positive rotation in acid solution (3). Correspondingly, the homocystine isomer with a specific rotation of  $+77^\circ$  in HCl possesses a rotation of  $-16^\circ$  in water. We shall therefore designate this isomer, which we have shown to be related configurationally to *l*-methionine, as *l*-homocystine. Since all the naturally occurring amino acids whose configurations have been established have been shown to belong to the same series, the above notation will harmonize as well with the system by which all members of this series are designated *l*-amino acids regardless of the observed direction of rotation (7).

#### EXPERIMENTAL

*Preparation of dl-S-Benzylhomocysteine*—5 gm. of inactive homocystine were added to approximately 50 cc. of dry liquid  $\text{NH}_3$  in a 3-neck flask fitted with a mercury seal mechanical stirrer and a  $\text{CaCl}_2$  tube. The flask was immersed in a cooling mixture of  $\text{CO}_2$  and ether. Sodium was added to the flask in small portions until a very slight excess was indicated by the persistence of the blue color. To this solution 5 cc. of benzyl chloride were slowly added. After removal of the excess  $\text{NH}_3$  the residue was dissolved in water and the solution neutralized with HCl. The precipitate was filtered, washed with water, and finally with alcohol. 8 gm. of the crude *dl*-*S*-benzylhomocysteine, representing 95 per cent of the theoretical yield, were obtained. The product was sufficiently pure to proceed with the formylation. The recrystallized product melted at  $240\text{--}250^\circ$  (corrected) with previous darkening, which is higher than that previously reported by Butz and du Vigneaud (4).

*Preparation of N-Formyl-dl-S-Benzylhomocysteine*—A mixture of 25 gm. of *dl*-*S*-benzylhomocysteine with 200 cc. of 85 to 90 per cent formic acid was warmed to  $50\text{--}60^\circ$  and 70 cc. of acetic anhydride were added from a separatory funnel at such a rate that the

liberated heat maintained a temperature of approximately 60°. The solution was then allowed to cool and 70 cc. of water were added to decompose any remaining acetic anhydride. The solution was concentrated *in vacuo*, the resulting oil was dissolved in 25 cc. of hot acetone, and 100 cc. of benzene were then added. After standing in the ice box overnight, the precipitate was filtered. The filtrate was concentrated to 50 cc. and a further quantity obtained. The combined precipitates amounted to 23.6 gm. of colorless plates melting at 85–86° (corrected), representing a yield of 84 per cent of the theoretical. The product for analysis was dried over  $P_2O_5$  at 61° *in vacuo* and had the following composition.

4.399 mg. substance: 0.214 cc. N at 27° and 760 mm.

0.1277 gm. " : 0.1198 gm.  $BaSO_4$  (Parr bomb)

$C_{17}H_{15}O_3NS$ . Calculated. N 5.53, S 12.66

Found. " 5.53, " 12.88

*Resolution of N-Formyl-dl-S-Benzylhomocystine*—10 gm. of N-formyl-dl-S-benzylhomocystine were dissolved in 15 cc. of methyl alcohol at room temperature and to this solution were added 15.7 gm. of anhydrous brucine in 15 cc. of methyl alcohol. The mixture was cooled in an ice-salt bath and the precipitate was filtered and washed with small portions of cold methyl alcohol. The precipitate amounted to 12 gm. and possessed a rotation of  $[\alpha]_D^{20} = -13^\circ$  for a 1 per cent solution in methyl alcohol. A further amount of 1 gm. was obtained by adding 50 cc. of ethyl acetate to the above filtrate and allowing the solution to remain in the ice box overnight. The separation of this brucine salt was sufficiently complete so that the mother liquor could be worked up directly for the other isomer as will be described later. The above precipitates were combined and recrystallized from 130 cc. of methyl alcohol. A yield of 9 gm. of product with a rotation of  $[\alpha]_D^{20} = -16^\circ$  was obtained. Another recrystallization raised the specific rotation to  $[\alpha]_D^{20} = -17^\circ$ , which remained constant upon further recrystallization. The compound after being dried over  $P_2O_5$  at 100° melted at 125–128° and had the following composition.

3.968 mg. substance: 0.237 cc. N at 25° and 759 mm.

0.1429 gm. " : 0.0511 gm.  $BaSO_4$  (Parr bomb)

$C_{16}H_{14}O_3N_2S$ . Calculated. N 6.49, S 4.95

Found. " 6.83, " 4.91

*Isolation of S-Benzyl-d-Homocysteine*—19.3 gm. of the dried brucine salt of N-formyl-S-benzyl-d-homocysteine were shaken with 50 cc. of chloroform and 100 cc. of 1 N  $\text{NH}_4\text{OH}$ , the aqueous layer was further extracted with two 10 cc. portions of chloroform, and then concentrated to about 75 cc. *in vacuo* in order to remove the excess  $\text{NH}_3$ . The resulting solution was neutralized to litmus with HCl and then sufficient HCl added to make the solution 1 N. After being refluxed for 1 hour, the reaction mixture was neutralized to litmus in the cold with  $\text{NH}_4\text{OH}$ . 6.4 gm. of S-benzyl-d-homocysteine, representing 96 per cent of the theoretical amount expected, were obtained. This product can be used directly for the conversion to d-homocysteine. The compound purified by recrystallization from water melted at  $247\text{--}252^\circ$  (corrected) with previous darkening and possessed a rotation of  $[\alpha]_D^{26} = -25^\circ$  for a 1 per cent solution in 1 N HCl. The product had the following composition.

4.394 mg. substance: 0.237 cc. N at  $23^\circ$  and 753 mm.

$\text{C}_{11}\text{H}_{15}\text{O}_2\text{NS}$ . Calculated, N 6.22; found, N 6.16

*Conversion of S-Benzyl-d-Homocysteine to d-Homocysteine*—6.4 gm. of S-benzyl-d-homocysteine dissolved in 40 cc. of liquid  $\text{NH}_3$  were treated with a slight excess of sodium. The  $\text{NH}_3$  was allowed to evaporate spontaneously and the residue was dissolved in 60 cc. of water. 0.1 gm. of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was added and air passed through the solution until the nitroprusside test for the sulfhydryl group was negative. The precipitate of ferric hydroxide was then filtered and the clear solution neutralized to litmus with HCl. 2.85 gm. of d-homocysteine were obtained, representing 75 per cent of the theoretical yield from the benzyl derivative. This product had a rotation of  $[\alpha]_D^{26} = -73^\circ$  for a 1 per cent solution in 1 N HCl. Two recrystallizations from water raised the rotation to  $[\alpha]_D^{26} = -77^\circ$ . The specific rotation of a 0.06 per cent solution in water was  $[\alpha]_D^{21} = +16^\circ$ . The product melted with decomposition at  $281\text{--}284^\circ$  (corrected) and had the following composition.

3.248 mg. substance: 0.298 cc. N at  $23^\circ$  and 760 mm.

$\text{C}_3\text{H}_7\text{O}_2\text{N}_2\text{S}_2$ . Calculated, N 10.44; found, N 10.58

*Isolation of l-Homocysteine*—The original mother liquors from the crystalline brucine salt of N-formyl-S-benzyl-d-homocysteine

were concentrated to 30 cc. and then decomposed as described above for the preparation of S-benzyl-*d*-homocystine. The conversion of the resulting S-benzyl-*l*-homocystine to *l*-homocystine was carried out in the same manner as described for *d*-homocystine. The crude *l*-homocystine had a rotation of  $[\alpha]_D^{26} = +55^\circ$  in HCl. A few recrystallizations from water yielded a product with a rotation of  $[\alpha]_D^{26} = +77^\circ$  for a 1 per cent solution in 1 N HCl and a rotation of  $[\alpha]_D^{21} = -16^\circ$  for a 0.06 per cent solution in water. The yield of the *l*-homocystine was approximately the same as that obtained for *d*-homocystine, based on the original inactive material.

*Conversion of Active Isomers of Homocystine to Corresponding Methionines*—0.16 gm. of the above *l*-homocystine was converted to methionine by reduction and subsequent methylation according to the procedure described by du Vigneaud, Dyer, and Harmon (8). 0.08 gm. of methionine was obtained, possessing a rotation of  $[\alpha]_D^{25} = -7.7^\circ$  for a 1 per cent solution in water. The methionine so obtained was identical with that of an authentic sample of naturally occurring methionine.

The *d*-homocystine was likewise converted to the corresponding methionine, yielding a product with a rotation of  $[\alpha]_D^{26} = +8.0^\circ$  in H<sub>2</sub>O and a rotation of  $[\alpha]_D^{25} = -22^\circ$  for a 1 per cent solution in 0.2 N HCl.

#### SUMMARY

The preparation of both optically active isomers of homocystine has been presented. N-Formyl-*dl*-S-benzylhomocystine was resolved by means of brucine, and the optically active benzylhomocysteines obtained from the brucine salts of the formyl derivatives were converted to the corresponding homocystines by scission of the benzyl group by sodium in liquid ammonia and oxidation of the resulting sodium homocysteinates. Both isomers were obtained in a high degree of purity and in excellent yield.

The optical isomers of homocystine so obtained were converted to the corresponding methionines. The homocystine, possessing a specific rotation of  $[\alpha]_D^{26} = +77^\circ$  in hydrochloric acid solution and a rotation of  $[\alpha]_D^{26} = -16^\circ$  in aqueous solution, yielded *l*-methionine identical with the naturally occurring methionine. This isomer of homocystine which we have designated *l*-homo-

cystine therefore corresponds in spatial configuration to the naturally occurring series. This was confirmed by the conversion of naturally occurring methionine to this same enantiomorph of homocystine. The *d*-homocystine was also converted to *d*-methionine.

The magnitude of the rotations of the methionines obtained from the active homocystines was as great as either that of *l*-methionine isolated from casein by enzymatic hydrolysis or that of the *l*- and *d*-methionines obtained in the resolution of *dl*-methionine, thus demonstrating the completeness of the resolution of homocystine which has been presented.

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## EVIDENCE OF ADSORPTION EXPERIMENTS ON THE FORMS OF CALCIUM AND INORGANIC PHOSPHORUS IN BLOOD SERUM

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It has been known, since the pioneer investigation of Rona and Takahashi (1), that the calcium of the blood stream exists in more than one form. Starting from this investigation there have been developed well defined procedures for fractionating the serum calcium into a diffusible and a non-diffusible component either by ultrafiltration or by compensation dialysis through collodion membranes. Many investigators assume that the calcium is present in more than one chemical state in both the diffusible and non-diffusible fractions of the serum.

In the present communication there will be mainly considered the claims for the existence of more than one form of calcium in the diffusible fraction. In this fraction, it has been proposed by Greenwald, Sendroy and Hastings, Klinke, Brull, and others (see (2) for literature) that there is present, besides ordinary calcium ion, an unknown organic citrate-like calcium compound. According to the work of one of us and L. D. Greenberg (2) there is no good positive evidence for the existence of such a compound. However, there also is no evidence which completely rules out its existence.

Since the appearance of the above paper, Benjamin and Hess (3-5) have published the results of a study of the adsorbent action of  $\text{BaSO}_4$  on serum. From this work they conclude that calcium is present in at least four forms in normal serum, two diffusible and two non-diffusible. They found no evidence for an organic calcium compound, but instead concluded that besides calcium ion, the diffusible calcium consists of a specific calcium-phosphorus

complex which experimentally is characterized by being adsorbable. According to them, calcium ion as such is not adsorbable by  $\text{BaSO}_4$ . The presence of this adsorbable calcium-phosphorus substance is not limited to blood since they state that it is spontaneously formed in artificial salt solutions that are buffered at a pH below neutrality. Benjamin and Hess attribute important physiological functions to this adsorbable compound in normal bone calcification and believe that it is abnormally low and therefore the cause of the faulty calcification in rickets. Because of these important implications, it is desirable carefully to examine the basis of the claims for the existence of the adsorbable calcium phosphate. Our adsorption experiments were initiated to investigate the claims for the citrate-like organic calcium compound. With the appearance of the above publications, the experiments were extended to cover the adsorbable calcium-phosphorus complex. The results obtained, in our estimation, do not support the claims for the existence of any special form of diffusible calcium other than ordinary calcium ion.

#### EXPERIMENTAL

The experiments were carried out on blood serum, the ultrafiltrates of blood serum, and on artificial serum solutions prepared from the inorganic components in the proportion in which they are present in blood. Sealed tubes containing the liquid and adsorbent were agitated in a mechanical shaker for periods of 3 hours or longer. The adsorbents employed were  $\text{BaSO}_4$ , kaolin, and permutit. These were prepared in the manner given below.

*BaSO<sub>4</sub> Preparation 1*—This was prepared, as described by Benjamin and Hess, from  $\text{BaNO}_3$  and  $\text{H}_2\text{SO}_4$ . The precipitate was dried in an oven at  $100^\circ$ .

*BaSO<sub>4</sub> Preparation 2*—Equivalent amounts of hot dilute solutions of  $\text{Na}_2\text{SO}_4$  and  $\text{BaCl}_2$  were mixed. The precipitate was filtered, washed with water and alcohol, then dried by washing with ether.

*BaSO<sub>4</sub> Preparation 3*—This was prepared by mixing equivalent portions of cold concentrated  $\text{Na}_2\text{SO}_4$  and  $\text{BaCl}_2$  solutions. The finely divided precipitate was washed with dilute  $\text{NaCl}$  solution by centrifugation and then dried with alcohol and ether.

*BaSO<sub>4</sub> Preparation 4*—This was prepared by pouring 1 mole of cold saturated  $\text{BaCl}_2$  into 2 moles of cold saturated  $\text{Na}_2\text{SO}_4$  solu-

tion. This forms a coagulum which breaks up into an extremely finely divided suspension on vigorous agitation. It is necessary to wash the precipitate by decantation with dilute NaCl in order to prevent a complete colloidal dispersion which is produced if it is washed with distilled water. After being washed with large quantities of 1 per cent NaCl solution until no further test for sulfate is obtained, the adsorbent is placed on a filter and dried by washing with alcohol and ether.

*BaSO<sub>4</sub> Preparation 5*—This was prepared on the same principle as Preparation 4, but with the idea of building up a Ba layer on the crystal surface. In this preparation, 1 mole of Na<sub>2</sub>SO<sub>4</sub> solution is poured into 2 moles of BaCl<sub>2</sub> solution. In washing this precipitate, 1 per cent NaCl solution and then alcohol were used to prevent colloidal dispersion. The preparation was then dried with alcohol and ether.

*Permutit*—This was washed with distilled water and dried *in vacuo*.

*Kaolin*—Kaolin was prepared by washing with 1 N HCl, then water, and drying *in vacuo*.

The analytical methods used in this investigation were Kirk and Schmidt's (6) for calcium, Fiske and Subbarow's (7) for phosphate, and Morgulis and Hemphill's (8) for sulfate. pH measurements were carried out by means of the glass electrode.

The few electro-osmosis experiments to determine the electrical charge of certain of the BaSO<sub>4</sub> adsorbents were carried out in a cell consisting of a U-tube, the two arms of the tube being separated by a 4 mm. bore stop-cock. In carrying out a determination the stop-cock bore was tightly packed with the BaSO<sub>4</sub> to be tested and the solution introduced to an equal height in each arm of the cell. First, however, the BaSO<sub>4</sub> was shaken with a portion of the test solution. A 110 volt direct current was led in through platinum electrodes and the direction of the water osmosis was determined by the changes in level of the solution produced in the two arms of the cell.

#### *Evidence of Adsorption on Claims for a Citrate-Like Complex Calcium Ion in Blood Serum*

The results of adsorption experiments have been offered as proof of the presence of a negatively complex calcium ion in the blood. Thus Klinke (9) states that, while such positive adsorbents as

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$\text{Ca}_3(\text{PO}_4)_2$ , bone,  $\text{BaSO}_4$ , and  $\text{MgNH}_4\text{PO}_4$  remove calcium from blood serum, the negative adsorbents, kaolin and animal charcoal, do not. This alleged evidence can be readily shown to be without foundation.  $\text{BaSO}_4$  adsorbs calcium from artificially prepared salt solutions containing no organic material. This has been shown by Benjamin (4) and was readily demonstrated by us.

The claim that negatively charged adsorbents will not adsorb calcium from serum also is not correct for all of them. In Table

TABLE I  
*Adsorption of Calcium from Serum and Serum Ultrafiltrates by Certain Negatively Charged Adsorbents*

Adsorbent	Fluid	Amount of adsorbent	Calcium		Phosphate		$\frac{\text{Ca adsorbed}}{\text{P adsorbed}}$
			Initially present	Adsorbed	Initially present	Adsorbed	
		gm. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	
Permutit	Serum A	3	10.8	6.4	6.3	0.8	8.0
		10	10.8	8.7	6.3	2.3	3.8
		30	10.8	9.8	6.3	3.6	2.7
	Ultrafiltrate of Serum A	3	7.0	5.1	6.5	0.7	7.3
		10	7.0	6.2	6.5	1.9	3.3
		30	7.0	6.3	6.5	3.2	1.9
Kaolin (acid-washed)	Serum A	3	10.8	0.1	6.3	0.8	0.1
		10	10.8	1.0	6.3	1.2	0.8
		30	10.8	3.8	6.3	1.8	2.1
	Ultrafiltrate of Serum A	3	7.0	0.6	6.5	0.1	6.0
		10	7.0	1.3	6.5	0.5	2.6
		30	7.0	2.6	6.5	1.9	1.3

If there are presented the adsorption results obtained on beef serum and the ultrafiltrates from the same serum. It is seen from Table I that both kaolin and permutit, which are negative adsorbents, have removed calcium from the serum and from the ultrafiltrate. Also, they adsorb phosphate. These data do not bear out the claim that only positive adsorbents remove calcium from blood serum.

To test the influence of a negatively charged complex on adsorption, some experiments were carried out with solutions containing citrate. These are given in Table II. When citrate is present in

considerable amount, as is seen from Table II, instead of calcium being more readily adsorbed by the positive adsorbent  $\text{BaSO}_4$ , no adsorption at all is obtained as long as phosphate is absent. When phosphate is added to the same solution, some adsorption of calcium takes place, but not nearly to the same extent as from citrate-less solutions.

The concentration of citrate is of importance in connection with this effect, as is shown in Table II. In this experiment the adsorption by  $\text{BaSO}_4$  and kaolin was tested on an inorganic serum

TABLE II  
*Adsorption with  $\text{BaSO}_4$  Preparation 3 from Inorganic Serum Solutions Containing Citrate*

Inorganic serum solution	Amount of adsorbent	Calcium		Phosphate	
		Initially present	Ad-sorbed	Initially present	Ad-sorbed
	gm. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
Buffered with 0.05 M citrate, pH 6.7 No phosphate or bicarbonate	10	12.6	0	0	
	30	12.6	0	0	
Phosphate added	10	12.6	0.7	11.1	0
	30	12.6	2.0	11.1	0
Buffered with bicarbonate, 1 mole citrate added per mole Ca present No phosphate	10	7.2	2.1	0	
	30	7.2	3.2	0	
Phosphate added	10	6.8	3.1	8.3	2.0
	30	6.8	4.7	8.3	3.4

solution containing citrate in the proportion of 1 mole of citrate to 1 mole of calcium. Both with phosphate absent and present,  $\text{BaSO}_4$  removes calcium to a lesser extent than in the absence of citrate. However, just as was noted by Greenberg and Greenberg with the electrodialysis method, detection of a small quantity of a citrate-like calcium compound in blood is not feasible with any of the above adsorbents.

#### *Adsorbable Calcium-Phosphorus Complex of Benjamin and Hess*

- An analysis of the evidence for the existence of the special adsorbable calcium-phosphorus complex proposed by Benjamin and

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Hess will now be undertaken.<sup>1</sup> Among the arguments advanced by these authors in favor of their thesis, the most important are those given below. (1) Adsorption of calcium does not take place from solutions of such simple salts as the chloride, phosphate, and gluconate of calcium, so presumably calcium ion, as such, is not adsorbable. Adsorption of calcium in the presence of phosphate does take place when the solution is buffered at an alkaline pH by such substances as bicarbonate, sodium *o*-nitrophenol, sodium diethylbarbiturate, pyridine, and aniline. (2) Barium sulfate removes an approximately constant amount of calcium and phosphate from normal serum or ultrafiltrates, leaving a residue of calcium in the ultrafiltrate which agrees with the values in the literature for the ionic calcium of the serum. (3) The residue of calcium left behind after one adsorption by barium sulfate is not adsorbed by the immediate addition of fresh adsorbent, a supposed demonstration that the adsorbable calcium phosphate has been completely removed and the ionic calcium in the residue is not adsorbable. However, after a lapse of 24 hours, more of the calcium becomes adsorbable.

We do not question the experimental data of Benjamin and Hess—indeed we have confirmed most of their findings. However, to confirm their observations, it is necessary to use a BaSO<sub>4</sub> adsorbent prepared essentially according to the directions of these authors; namely, from a sulfuric acid solution. In our estimation, quite a different interpretation can be placed upon their experiments than the one which these authors give. Our view-point is based on the experimental evidence given below which indicates that the adsorptive behavior of BaSO<sub>4</sub> preparations depends upon (1) the method of preparation, (2) the nature of the anions in the solution, and (3) the pH of the solution.

In the first instance, exception may be taken to the widely prevalent view that only one-third to one-half of the diffusible calcium of the blood is present as calcium ions. A critical examina-

<sup>1</sup> While the analysis in the text is confined to the evidence for the existence of an ultrafiltrable calcium-phosphorus complex, it appears, particularly from the work of Benjamin and Hess on hypercalcemic sera (5), that no specific fractionation of the non-diffusible components is obtained by BaSO<sub>4</sub> adsorption. This view is based on the widely varying ratios of adsorbed calcium to phosphorus reported.

tion of the experimental measurements offered in support of this low level for the content of calcium ions in the blood (see (2)) shows that most of the experimental methods which have been employed for this purpose are objectionable, and that the results obtained by them have little weight. Evidence which favors a level of ionic calcium nearly equivalent to the values for the diffusible calcium of biological fluids has recently been offered by McLean and Hastings (10). The method used by these authors was based on the effect of calcium on the contraction response of the isolated frog heart.

Secondly, all  $\text{BaSO}_4$  preparations do not show the same activity and it is readily possible to prepare  $\text{BaSO}_4$  adsorbents of varying degrees of efficiency, ranging from ones which only have a small calcium adsorptive power to others with a higher efficiency than the  $\text{BaSO}_4$  of Benjamin and Hess. Coarse precipitates formed from hot dilute solutions, as might be expected from known colloidal principles, were found to be poorer adsorbents than finely divided precipitates prepared from concentrated cold solutions. Further, on similar lines, an adsorbent prepared from an excess of sulfate ion is more effective in adsorbing the cation calcium, and one prepared from barium excess is more efficient for phosphate. In this way the amounts of calcium and phosphate adsorbed can readily be altered. These factors are well illustrated by the data given in Table III.

In the adsorptive series from artificial serum solutions given in Table III,  $\text{BaSO}_4$  preparations embodying all the factors just mentioned have been employed.  $\text{BaSO}_4$  Preparation 2, prepared from dilute hot solutions, is the poorest adsorbent on the list for both calcium and phosphate; Preparation 1, prepared according to the method of Benjamin and Hess, is very effective for both calcium and phosphate; Preparation 3, from cold concentrated solutions, is nearly as good as Preparation 1. However, Preparation 4, prepared from sulfate excess, removed calcium almost to the limit of analytical detection, but only moderately adsorbed phosphate, while Preparation 5, prepared from an excess of barium, removed the phosphate completely. The particular amount of adsorption obtained by Benjamin and Hess, it is seen from this series, does not have the significance they assume, but merely appears to be the result of a chance preparation of a particular adsorbent. From



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our list of adsorbents, the activity of BaSO<sub>4</sub> Preparation 4 and, in some instances, permutit, which removed practically all of the calcium from serum, serum ultrafiltrates, or inorganic solutions, although leaving behind a considerable proportion of the phosphate, can hardly be fitted to the Benjamin and Hess theory. To do so would require that practically none of the calcium be present as the ion in the serum or the inorganic solutions.

TABLE III\*  
*Adsorption from Inorganic Serum Solutions\**

Adsorbent	Amount of adsorbent	Calcium		Phosphate		Ca adsorbed P adsorbed
		Initially present	Adsorbed	Initially present	Adsorbed	
	gm. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	
Kaolin	10	9.0	3.8	8.8	3.8	1.0
	30	9.0	4.8	8.8	7.4	0.6
Permutit	10	9.4	6.9	8.7	3.7	1.9
BaSO <sub>4</sub> Preparation 1	10	11.0	8.7	8.5	5.0	1.7
	30	11.0	9.7	8.5	6.2	1.6
BaSO <sub>4</sub> Preparation 2	10	11.0	4.0	8.5	2.1	1.9
	30	11.0	6.5	8.5	4.4	1.5
BaSO <sub>4</sub> Preparation 3	10	11.0	5.8	8.5	3.1	1.9
	30	11.0	7.5	8.5	4.9	1.5
BaSO <sub>4</sub> Preparation 4	10	9.8	8.4	8.4	4.2	2.0
	30	9.8	9.4	8.4	4.4	2.1
	40	10.2	9.8	5.5	2.8	3.4
BaSO <sub>4</sub> Preparation 5	40	9.9	7.4	5.5	5.5	

\* The inorganic serum solutions used in these experiments were prepared to contain 80 mm of NaCl, 75 mm of NaHCO<sub>3</sub>, 4 mm of KCl, and 3 mm of K<sub>2</sub>SO<sub>4</sub> per liter. Calcium chloride and dipotassium phosphate were added in the amounts desired. The pH of each solution was adjusted to 7.4 by bubbling in CO<sub>2</sub>.

While with the BaSO<sub>4</sub> adsorbent prepared by the method of Benjamin and Hess no calcium is adsorbed from simple calcium salt solutions, by the principles just given, it is possible to prepare BaSO<sub>4</sub> powders that will readily adsorb calcium even from these. Such a series of experiments is given in Table IV, where the adsorption of BaSO<sub>4</sub> Preparations 1 and 4 and permutit was tested on

dilute solutions of the chloride, lactate, gluconate, and phosphate of calcium.  $\text{BaSO}_4$  Preparation 1 removed no calcium. On the other hand, both  $\text{BaSO}_4$  Preparation 4 and the permutit removed the calcium from these solutions almost to completeness. Calcium ion as such, then, can be adsorbed by some  $\text{BaSO}_4$  and other adsorbents.

Not only the method of preparation, but also the nature of the anions present in the solution, markedly affects the adsorption of

TABLE IV  
*Adsorption from Dilute Solutions of Calcium Salts*

Adsorbent	Salt	Original Ca concentration	Ca after adsorption	pH	
				Before adsorption	After adsorption
		mg. per 100 ml.	mg. per 100 ml.		
$\text{BaSO}_4$ Preparation 1 (40 gm. per 100 ml.)	$\text{CaCl}_2$	10.3	10.5	6.50	4.00
	" in 0.1 N NaOH	11.6	10.0		
	" " 0.1 " $\text{H}_2\text{SO}_4$	9.1	7.7		
	" " 0.1 per cent $\text{Na}_2\text{SO}_4$	11.5	9.6		
	Ca lactate	6.4	6.7	6.57	4.16
$\text{BaSO}_4$ Preparation 4 (30 gm. per 100 ml.)	" gluconate	8.2	8.4	6.32	4.18
	$\text{CaCl}_2$	15.0	0.2	6.50	8.67
	" in 0.05 M HCl	8.0	1.7		
	Ca lactate	8.2	0.3	6.57	8.56
	" gluconate	5.8	0.2	6.33	8.33
Permutit (30 gm. per 100 ml.)	" phosphate ( $\text{CaHPO}_4$ )	5.0	0.0		
	$\text{CaCl}_2$	16.1	0.2		
	Ca lactate	22.1	0.2		
	" gluconate	14.6	0.2		

The  $\text{BaSO}_4$  adsorbents have the following pH in distilled  $\text{H}_2\text{O}$ : Preparation 1, 4.0; Preparation 4, 8.62; Preparation 5, 3.96.

calcium by  $\text{BaSO}_4$  and also by other adsorbents. An insight into the reason for this is offered by the work of Michaelis and Dokan (11) on the influence of ions on the electrical charge of  $\text{BaSO}_4$ . By electro-endosmose experiments, these authors showed that  $\text{BaSO}_4$  precipitates usually carry a positive electrical charge. With the exception of hydrogen and hydroxyl ions, univalent ions influence this charge very little. Hydrogen ions markedly increase the

positivity of the charge and hydroxyl ions are capable of reversing the sign of the charge of  $\text{BaSO}_4$  from positive to negative. However, ions of higher valency strongly affect the charge, and bivalent anions too are readily capable of reversing the sign of the adsorbent from positive to negative. While hydrogen ions tend to increase the degree of positiveness of the charge, their effect may be outweighed by anions of higher valence. As an example of this,  $\text{BaSO}_4$ , when placed in dilute sulfuric acid solution, is found to assume a negative charge.

The adsorption of calcium by  $\text{BaSO}_4$  parallels the changes produced in the electrical charge. Adding phosphate or increasing

TABLE V

*Independent Adsorption with  $\text{BaSO}_4$  Preparation 3 of Calcium and Phosphate from Inorganic Serum Solutions*

	Amount of adsorbent	Calcium		Phosphate	
		Initially present	Ad-sorbed	Initially present	Ad-sorbed
	gm. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
Buffered with 0.01 M borate, pH 7.60, no phosphate or $\text{NaHCO}_3$	10	7.6	0.8		
	30	7.6	1.7		
Phosphate added	10	7.6	2.4	6.0	2.6
	30	7.6	3.7	6.0	3.8
Buffered with 0.05 M potassium acid phthalate, pH 4.0	10	6.1	0	8.1	0
	30	6.1	0.9	8.1	0.1

the phosphate concentration of a solution increases the amount of calcium which is adsorbed. Ample evidence of this is offered in the work of Benjamin and Hess. Our own experiments, shown in Table IV, demonstrate that the  $\text{BaSO}_4$  of Benjamin and Hess, listed here as Preparation 1, which does not adsorb calcium from aqueous solutions of simple calcium salts, does adsorb calcium from calcium chloride dissolved in dilute solution of  $\text{NaOH}$ ,  $\text{Na}_2\text{SO}_4$ , or even  $\text{H}_2\text{SO}_4$ .

From Table V it is seen that even  $\text{BaSO}_4$  preparations which are capable of adsorbing calcium from solutions containing no phosphate ions show an increase in the amount of calcium adsorbed, if phosphate is present and the solution is buffered on the alkaline side of neutrality.

The fact that other polyvalent ions can also favor the adsorption of calcium is strong evidence that the formation of a peculiar calcium-phosphorus compound is not a necessary condition for the adsorption of calcium by any  $\text{BaSO}_4$  preparation. In Table VI there are tabulated experiments which demonstrate the capacity of sulfate ion to act in this manner. In these experiments it is

TABLE VI

*Influence of Sulfate on Adsorption of Calcium Inorganic Serum Solution Containing No Phosphate, Buffered with Bicarbonate at pH 7.4*

	Amount of adsorbent	Calcium		Sulfate	
		Present	Ad-sorbed	Present	Ad-sorbed
	<i>gm. per 100 ml.</i>	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>
Constant sulfate content, varying amounts $\text{BaSO}_4$ Preparation 1	0	8.0	0	47.3	0
	10	6.2	1.8	45.2	2.1
	20	5.8	2.2	44.9	2.4
	30	5.8	2.2	42.4	4.9
	40	5.6	2.4	40.4	6.9
	60	5.3	2.7	38.1	9.2
	80	5.0	3.0	35.1	12.2
	100	4.2	3.8	32.2	15.1
	Sodium sulfate added to solution				
	<i>per cent</i>				
Varying sulfate content, constant amount $\text{BaSO}_4$ Preparation 1 (40 gm. per 100 ml.)	0	9.5	0		0
	0.10	7.8	1.7		5.6
	0.20	7.8	1.7		7.0
	0.40	7.9	1.6		7.0
	0.50	7.8	1.7		6.8

shown that  $\text{BaSO}_4$  Preparation 1, which does not adsorb calcium from solutions of calcium salts of univalent anions, acquires the capacity to adsorb calcium if the bivalent sulfate ion is added to the solution. It is true that the sulfate ion is not quite so effective in this respect as is the corresponding phosphate ion, probably because phosphate ion is more readily adsorbable. Paralleling the behavior of this adsorbent in phosphate-containing solutions, there is an adsorption of sulfate as well as of calcium from solutions

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in which sulfate replaces phosphate. As has already been pointed out, calcium is adsorbed by  $\text{BaSO}_4$  Preparation 1 from solutions of calcium chloride containing  $\text{NaOH}$ ,  $\text{Na}_2\text{SO}_4$ , or  $\text{H}_2\text{SO}_4$ .

The influence of hydrogen ion is somewhat subsidiary to the other factors which have been discussed. Depending upon their preparation, the different  $\text{BaSO}_4$  adsorbents show either an acid or alkaline reaction in solution. Preparation 1 changes the reaction of the solutions into which it is introduced to a greater acidity. In the solutions of the calcium salts in the experiments of Table IV, the acid shift is from about pH 6.5 to 4.0.  $\text{BaSO}_4$  Preparation 5, prepared from a barium excess, also shows a residual acidity. In distilled water it developed the pH of 3.96. On the other hand,

TABLE VII  
*Effect of Adsorption on pH of Serum and Inorganic Serum Solutions*

Solution	$\text{BaSO}_4$ preparation No.	pH	
		Before adsorption	After adsorption
Beef serum.....	1	7.51	7.44
Inorganic serum solution.....	1	7.60	7.42
Beef serum.....	2	7.48	7.35
Inorganic serum solution.....	2	7.57	7.48
“ “ “.....	3	7.60	7.46
Beef serum.....	4	7.48	7.52
“ “ “.....	5	7.51	7.13

$\text{BaSO}_4$  Preparation 4, prepared from sulfate excess, produces an alkaline shift in dilute calcium salt solutions from about pH 6.5 to 8.5.

The pH of blood serum and inorganic serum solutions is also shifted by the  $\text{BaSO}_4$  adsorbents. The magnitude of this effect is illustrated in Table VII. All the  $\text{BaSO}_4$  preparations, except Preparation 4, cause a shift of pH toward greater acidity in serum or inorganic serum solutions. This is true of adsorbents prepared from equivalent quantities of the neutral salts,  $\text{BaCl}_2$  and  $\text{Na}_2\text{SO}_4$ , as well as the powder prepared from acid solution or an excess of  $\text{BaCl}_2$ . The mean change in pH produced by these adsorbents is around 0.15 unit.  $\text{BaSO}_4$  Preparation 5, prepared from barium excess, produced an alteration of about 0.35 pH unit. On the other

hand, Preparation 4, which has an alkaline reaction in water, caused a small alkaline shift in serum.

The residual acidity of the Benjamin and Hess  $\text{BaSO}_4$  preparation helps to explain why it does not adsorb calcium from dissolved  $\text{CaHPO}_4$ . The acidity from the  $\text{BaSO}_4$  Preparation 1 aids the hydrolysis of the secondary to the primary phosphate. This being a univalent anion influences the nature of the adsorption very little.

On the other hand, the favoring effect pointed out by Benjamin and Hess of bicarbonate and other alkaline buffers on the adsorption of calcium follows from the increase and the stabilization of the content of the higher valence phosphate ions, and probably also from a neutralization of hydrogen ions adsorbed on the  $\text{BaSO}_4$ . In solutions on the acid side of neutrality, the other  $\text{BaSO}_4$  preparations tend to acquire the characteristics of Preparation 1. Thus,  $\text{BaSO}_4$  Preparation 3, which readily adsorbs calcium and phosphate from solutions on the alkaline side of neutrality, adsorbs only a trace of calcium and no phosphate from a solution buffered with potassium acid phthalate at the pH of 4.0. This is shown in Table V. On the other hand,  $\text{BaSO}_4$  Preparation 4, because of its excess of sulfate, readily adsorbs calcium even from 0.05 M HCl solution (see Table IV).

#### DISCUSSION

The following hypothesis is offered as an attempt to explain the many different features observed with the individual adsorbents in various types of solutions. The explanation will be confined to the  $\text{BaSO}_4$  adsorbents, although the others can readily be fitted into a similar scheme.

From the work of Michaelis and Dokan, it would appear that monovalent ions are only very slightly adsorbed but bivalent ions and ions of higher valency are readily adsorbed by  $\text{BaSO}_4$  powders. Exceptions to this are hydrogen and hydroxide ions which are also strongly adsorbed. One would expect the common ions, barium and sulfate, to be quite strongly adsorbed by  $\text{BaSO}_4$ . The nature of the ions which are adsorbed determines the electric charge upon the  $\text{BaSO}_4$ . In the course of their formation, the  $\text{BaSO}_4$  powders acquire an adsorption layer which is dependent on the type of the solution from which they are prepared. This layer

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is all important since, in all likelihood, it determines the specific properties of each adsorbent. The subsequent behavior of each adsorbent, when equilibrated with various solutions, to a large extent, consists of an exchange between the ion components of the already adsorbed layer for other strongly adsorbed ions which may be present in a given solution. Some qualitative electro-osmosis measurements, carried out on certain of the  $\text{BaSO}_4$  preparations, may be offered in favor of the above statements.

$\text{BaSO}_4$  Preparation 1, it was observed, carried a strong positive charge when it was immersed in an inorganic serum solution buffered with bicarbonate at pH 7.4, but containing no calcium

TABLE VIII

*Further Adsorption Obtained on Addition of Fresh  $\text{BaSO}_4$  Preparation 1 (40 Gm. per 100 Ml.)*

The values are given in mg. per 100 ml.

Period	Calcium		Phosphate	
	Present	Ad- sorbed	Present	Ad- sorbed
Initial value.....	7.1	0	6.0	0
After 3 hrs. shaking.....	3.1	4.0	4.0	2.0
Liquid then divided into 2 portions				
Sample 1. Immediately shaken with fresh $\text{BaSO}_4$ .....	2.1	1.0	Trace	4.0
Sample 2. Kept for 24 hrs., then shaken..	1.8	1.3	"	4.0

or phosphate.  $\text{BaSO}_4$  Preparation 5 likewise was strongly positive under the same conditions. When phosphate equivalent to 5 mg. of P per 100 ml. was added to the solution, the charge on  $\text{BaSO}_4$  Preparation 1 became negative in sign. On the other hand,  $\text{BaSO}_4$  Preparation 4, in the absence of either calcium or phosphate, did not show a charge of sufficient magnitude to be detected with the apparatus which was employed.

The adsorption of calcium, it was found, is favored by the presence of phosphate or of sulfate. Adsorption of calcium ions alone leads the adsorbent to become increasingly positive in charge and so tends to inhibit the further adsorption of calcium. Similarly, adsorption of phosphate alone should lead to the inhibition of phosphate adsorption because of the increasing negativity. How-

ever, by the adsorption of the two ions together, there is a neutralization of the charge which makes it possible to increase the amount of each which is adsorbed through the building up of extended layers of alternately adsorbed calcium and phosphate.

An explanation appears from this reasoning for the last of the three important arguments that have been advanced for the existence of a specific adsorbable calcium phosphate; namely, the demonstration of a non-adsorbable residue believed presumably to consist of calcium and phosphate ions.<sup>2</sup> When by adsorption with one portion of BaSO<sub>4</sub> Preparation 1 the phosphate is reduced to a level too low further to appreciably affect the charge, no calcium will be adsorbed by a fresh portion of BaSO<sub>4</sub> because of the inhibiting effect of the positive charge it carries.

*Biochemical Significance*—If the failure of the Benjamin and Hess theory of the existence of a specific adsorbable calcium-phosphorus complex is granted, then their claims for an important relationship between the amount of adsorbable calcium with normal calcification and its aberration in rickets has to be revised. The findings of Benjamin on the calcification of cartilage comes to no more than the well known experiments of Shipley, Kramer, and Howland (12) with the substitution of an adsorption explanation. Shipley, Kramer, and Howland found that when the product of calcium and phosphorus expressed in mg. per cent was 40 or above, calcification readily takes place, but below a level of about 35 it does not. Benjamin no more than repeated this observation. While we do not subscribe to the view that the  $\text{Ca} \times \text{P}$

<sup>2</sup> We have not been able unequivocally to confirm the experimental evidence offered in this connection. With one preparation of BaSO<sub>4</sub> Preparation 1 there was obtained no further adsorption of calcium after a first adsorption either immediately or after 24 hours standing. With another preparation, however, there was obtained a further uptake of calcium of about the same magnitude on second adsorption both when carried out immediately or after a 24 hour period. The results of an experiment with the latter preparation, which was carried out on an inorganic serum solution of the composition used by Benjamin (4), are shown in Table VIII. Furthermore, the observation that calcium and phosphate are as readily adsorbed and apparently to the same extent from solutions to which calcium and phosphate have been freshly added, as from those of long standing, is difficult to reconcile with the deduction drawn by Benjamin that the alleged adsorbable calcium-phosphorus complex is only slowly formed.



product is a quantitative measure of calcification, it appears to us that a true solubility rather than adsorption is involved in calcification.

The lowered adsorption of calcium from rachitic serum is also readily explainable by the small amount of phosphate ion present without any need to postulate the absence of a specific compound. Increasing the phosphate content, it has been noted, leads to an increase in the amount of calcium adsorbed and a logical explanation for this has been offered in this discussion. On this reasoning the lowered phosphate in rickets would rationally lead to a decrease in calcium adsorption.

*Physicochemical States of Calcium and Phosphate in Serum—*The analysis in this paper and that in the previous publication by the senior author and L. D. Greenberg (2) show that a very large part of the evidence offered for the existence of biochemically important calcium compounds in the diffusible fraction of blood serum, other than ordinary calcium ion, is unsubstantial. This holds for both a citrate-like calcium compound and a specifically adsorbable calcium-phosphorus complex. It is true that the work given in these two papers does not categorically dismiss the possibility of such special calcium compounds, but rather shows that their existence has in no way been actually demonstrated. However, until such compounds, if they exist, are firmly established, it is more useful to attempt to explain the properties of blood on the basis of existing knowledge.

Until more substantial proof is offered for other substances, it is more useful to accept the existence of only calcium and phosphate ions in the diffusible component of blood serum. Even if low values of calcium ion activity in serum are admitted, it is qualitatively, at least, to be ascribed to an explanation familiar in connection with modern theories of strong electrolytes; namely, as the result of the depressing action of the interionic influence of the electrolytes and proteins of the blood.

#### SUMMARY

Adsorption experiments with variously prepared  $\text{BaSO}_4$  powders, permutit, and kaolin give no evidence for the existence of either a citrate-like calcium compound or for a specifically adsorbable calcium-phosphorus complex in blood serum. Instead, all of the

adsorption features which have been attributed to these hypothetical substances can be obtained under the proper conditions with merely calcium and phosphate ions.

It is preferable to accept the existence of only calcium and phosphate ions in the diffusible fraction of the serum until there is good evidence to the contrary.

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# THE FORMS OF THE CALCIUM AND INORGANIC PHOSPHORUS IN HUMAN AND ANIMAL SERA

## IV. A REPLY TO GREENBERG AND LARSON

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In their present paper (1), Greenberg and Larson have made an interesting study of the adsorptive properties of various types of barium sulfate. They have confirmed the fact that barium sulfate Preparation 1 (*i.e.* the preparation made according to our directions) does not ordinarily adsorb calcium ion. Under the conditions existing in serum, however, they believe it possible, on the basis of physicochemical principles, for removal of calcium ions by this adsorbent to occur. It is our opinion that in building up their explanation these authors have overlooked several important observations which make it impossible to accept such a theory.

In order to adhere to the sequence of Greenberg and Larson's paper we shall first consider the arguments against our hypothesis.

1. *Experiments Involving Use of Different Types of Barium Sulfate*—In our opinion experiments of this kind are irrelevant to the problem. They may be interpreted to mean simply that other types, in contrast to barium sulfate Preparation 1, are not useful in effecting a partition of the calcium in serum. Our point of view can, perhaps, be best illustrated by a consideration of the applicability of collodion membranes for this purpose. It is a simple matter to prepare membranes which allow all of the calcium to pass through, but this fact does not vitiate the conclusion that the calcium is present in both a filtrable and a non-filtrable form. Similarly, the properties exhibited by other types of barium sulfate need not concern us. The matter hinges entirely on the question, discussed in section (3), as to whether barium sulfate Preparation 1 possesses selective properties.

The answer likewise determines the position of this preparation

in the adsorptive series of Greenberg and Larson. It becomes a chance preparation with an intermediate adsorptive capacity only if it is proved that it is calcium ion which is adsorbed. Otherwise, it logically assumes a place at the bottom of the list. This possibility need not introduce any theoretical difficulty. The inability to remove calcium ion by exchange or polar adsorption does not necessitate an inability to adsorb a complex containing calcium, by a process of non-polar adsorption. The latter mechanism is perfectly well recognized in connection with the numerous examples of adsorption of non-ionic substances.

Mention should also be made of the second method by which Greenberg and Larson varied the adsorptive properties of their barium sulfate powders; namely, by increasing the size of the particles, as in barium sulfate Preparation 2. It is obvious that this does not affect the interpretation of our experiments, since in every case we used sufficient adsorbent to obtain *maximum* adsorption. Two barium sulfate preparations which differ only in the size of their particles should, under the proper conditions, remove the same amount of calcium. Since Greenberg and Larson failed to standardize their preparations, one cannot properly compare the amounts of calcium adsorbed.

2. *Experiments Involving Use of Polyvalent Anions Other Than Phosphate to Influence Adsorption of Calcium*—In the experiments of Greenberg and Larson, when barium sulfate Preparation 1 was placed in solutions which could reverse its positive charge (NaOH,  $\text{Na}_2\text{SO}_4$ , and  $\text{H}_2\text{SO}_4$ ) it became capable of adsorbing calcium from a solution of calcium chloride. Furthermore, in artificial ultrafiltrates containing no calcium or phosphate, the charge of this adsorbent became negative when phosphate was added to the solution. The authors reason that the presence of phosphate thus makes barium sulfate Preparation 1 capable of adsorbing calcium ion. However, the theoretical possibility that a negatively charged adsorbent may remove calcium ion does not necessarily mean that under a particular set of conditions it will do so. Other factors may come into play to prevent this. The conditions of Greenberg and Larson's experiments are not those obtaining in serum, in which calcium and phosphate are present *simultaneously*. Under these circumstances we have no evidence that phosphate is first adsorbed, and in amounts sufficient to reverse the charge on

the adsorbent. It is just as reasonable to assume that only sufficient phosphate ion is taken up to neutralize the charge, whereupon the calcium-phosphorus complex is adsorbed—or that both are removed at the same time. In other words, rather than establish the proof, this argument merely presents the possibility that barium sulfate Preparation 1 removes calcium ion from serum.

It should be mentioned that according to the figures of Greenberg and Larson, sulfate and phosphate do not seem to behave in the same manner. In the first place 15 mg. of *adsorbed* sulfate ( $15 \times 10^{-2}$  mM) caused the adsorption of only 3.8 mg. of calcium—an amount which can be removed in the presence of about 3 mg. ( $7 \times 10^{-2}$  mM) of *adsorbed* phosphorus. Moreover, an increase in the barium sulfate concentration up to 100 per cent resulted in a continuously increasing adsorption of both calcium and sulfate, whereas with phosphate a definite maximum is reached at 40 per cent, beyond which further addition of adsorbent has no effect.

3. *Evidence against Adsorption of Calcium Ion by Barium Sulfate Preparation 1*—Since there is no direct proof regarding the nature of the calcium in serum it is important to consider which theory best fits the known facts. Certain data, which will now be discussed, are in opposition to the theory that all of the filtrable calcium is in the ionic state.

When barium sulfate Preparation 1 was shaken with an artificial ultrafiltrate a definite, reproducible part of the calcium was adsorbed.<sup>1</sup> This was the same whether the barium sulfate was made from equivalent amounts of barium nitrate and sulfuric acid or from a 10-fold excess of either (Paper I (3), p. 32, foot-note 3). When sufficient barium sulfate was used (40 per cent) a maximum amount of calcium was removed, so that addition of a fresh portion of barium sulfate did not adsorb any more. *In no case was it possible to remove all of the calcium with this adsorbent.* The significance of these data becomes clear when one considers the reac-

<sup>1</sup> In Paper II (2), Table I, it was demonstrated that at the same pH the amount of calcium adsorbed increased with increasing concentration of  $\text{BHCO}_3$ . The high percentage of calcium adsorbed in the experiments of Greenberg and Larson (80 per cent) was undoubtedly due largely to the fact that the bicarbonate content in their solutions was 3 times that in serum. Another factor which would tend to increase the adsorption of calcium was the fact that the total calcium content was 11 mg., as in serum, rather than 6 mg., as in ultrafiltrates.

tion resulting from the addition of oxalate to serum. The oxalate removes the calcium ion by precipitation, whereupon the equilibrium between the types of calcium in the serum (*e.g.* that bound to protein) is immediately shifted and more calcium ion forms. The net result is that within a very few minutes all of the calcium in the serum is precipitated. In other words, the shift in equilibrium toward calcium ion is very rapid and complete, whereas conversion of calcium ion into the type adsorbable by barium sulfate Preparation 1 is relatively slow ((2) p. 60). It would, therefore, seem logical to assume that suitable amounts of other reagents which react with calcium ion in serum would, like oxalate, remove all of the calcium. On these grounds, the adsorption of virtually all of the calcium by barium sulfate Preparation 4 (a preparation which removes calcium ion from simple solutions) is not surprising. Conversely the inability of barium sulfate Preparation 1 to adsorb the calcium completely would, in itself, constitute an argument against the probability of adsorption of ionic calcium by this preparation.

Greenberg and Larson propose an explanation for this observation. According to their conception calcium cannot be adsorbed by barium sulfate Preparation 1 except in the presence of phosphate or some other polyvalent adsorbable anion. The process is believed by them to take place by means of the building up of alternately adsorbed layers of phosphate and calcium ions and the reason for the cessation of calcium adsorption is thought to lie in the reduction of the phosphate concentration to a level too low further to affect the charge. At this point addition of fresh barium sulfate is thought to be incapable of adsorbing the remaining calcium because of the inhibiting effect of the positive charge it carries. This contention is in direct contradiction to the experimental evidence. In Paper II ((2) p. 61) we stated:

"... it was also apparent that the failure of the fresh batch of barium sulfate to remove calcium and phosphorus from the solution when the second adsorption was carried out. . . was not due simply to the fact that these elements, after the first adsorption, were present in very small amounts (3.7 mg. of calcium and 3.7 mg. of phosphorus per 100 cc.). When an artificial ultrafiltrate was made which contained these amounts of calcium and phosphorus, shaking with barium sulfate resulted in appreciable adsorption."

Moreover, the fact that, in experiments such as that referred to above, the second lot of barium sulfate, though it removed no calcium, *always removed phosphorus* to the extent of about one-fourth to one-half the amount adsorbed by the first lot, cannot be reconciled with the explanation of Greenberg and Larson.

The lack of constancy of the ratio of Ca adsorbed to P adsorbed from different solutions by barium sulfate Preparation 1 constitutes another difficulty with the conception of Greenberg and Larson. If it were true that for a given amount of calcium adsorption a definite amount of phosphate must first be adsorbed (and *vice versa*), one would think that the ratio of Ca adsorbed to P adsorbed would always be the same. This is not the case. It seems more probable that phosphorus is adsorbed in two forms; ionic, and as part of the complex, the proportion of each being dependent on the composition of the solution.

According to the conception of Greenberg and Larson, increased acidity should result in diminished adsorption of calcium ion by barium sulfate Preparation 1. Indeed, their explanation for the fact that this adsorbent removes calcium from serum and not from a solution of  $\text{CaHPO}_4$  rests on the difference in pH of these fluids. As was pointed out by us ((2) pp. 59-60), however, the degree of adsorption by barium sulfate Preparation 1 is not influenced by changes in the pH of the artificial ultrafiltrate from 7.9 to 5.7. These experiments have since been repeated, with the same results<sup>2</sup> and constitute another argument against the belief that it is calcium ion which is adsorbed by barium sulfate Preparation 1. The favoring effect of bicarbonate and other alkaline buffers on adsorption could not have been due to increased alkalinity. All of these experiments were carried out at about pH 7.4. The increased adsorption of calcium can, therefore, hardly be accounted for on the basis of an increased content of higher valence phosphate ions or a neutralization of hydrogen ions on the surface of the barium sulfate.

Greenberg and Larson suggest that the calcium and phosphate ions are adsorbed by barium sulfate Preparation 1 largely by exchange with the adsorbed film of acid which is carried by this

<sup>2</sup> The determinations of pH, made with the glass electrode, were very kindly carried out by Mr. T. Rosebury of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University.



adsorbent. If this were the case, hydrogen and sulfate ions would be returned to the solution in exchange for adsorbed calcium and phosphate ions. As a result an increasing concentration of sulfuric acid should be built up in the solution. This does not occur. After the initial fall in pH, caused by the addition of the acid adsorbent to the solution, no further increase in acidity develops during the course of adsorption ((2) p. 64).

Finally, it should be mentioned that the method of McLean and Hastings (4) for the determination of calcium ion by the use of the frog's heart, which Greenberg and Larson mention as giving results in accordance with their theory, has not been demonstrated to be specific for calcium ion. The fact that the frog's heart does not react to calcium proteinate or to calcium citrate does not exclude the possibility that it does react to other complexes containing calcium.

4. *Biochemical Significance*.—On the basis of the preceding discussion, it seems to us that if all of the experimental evidence is considered, it becomes difficult to escape the conclusion that a calcium-phosphorus complex exists in serum. The adsorption theory of calcification thus becomes highly probable, particularly since the same fraction of calcium is removed by both barium sulfate Preparation 1 and cartilage.

Regarding the significance of the diminished adsorption of calcium from rachitic serum, the explanation offered by Greenberg and Larson would hold only in connection with the low phosphorus type of rickets. Their theory does not fit the data obtained with the low calcium type. In the latter form, in which the serum phosphate remains within normal limits while the total filtrable calcium is reduced, it should follow from their hypothesis that the proportion of adsorbable calcium should be increased and the unadsorbed residue reduced. The opposite is the case. The proportion of adsorbable calcium in the serum of rachitic puppies was found to be 38 per cent whereas the normal was 45 per cent. Moreover, the unadsorbed filtrable remainder was the same in both ((3) p. 47).

#### SUMMARY

The barium sulfate preparation used in our experiments gives a definite partition of serum calcium, and does not adsorb calcium

ion. The fact that other adsorbents (barium sulfate prepared in other ways) do not so behave does not invalidate this partition. The experimental data discussed support the hypothesis that the adsorbable calcium is in the form of a calcium-phosphorus complex of physiological importance.

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# THE PURIFICATION OF THE ENZYMES WHICH OXIDIZE CERTAIN AMINO ACIDS

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Broken cell suspensions of the livers and kidneys of various animals will oxidize alanine, proline, oxyproline, phenylalanine, and tyrosine (1-3). Certain other amino acids, serine, valine, leucine, isoleucine, and methionine, have now been tried and found to be similarly oxidized. Moreover, it has been possible to purify the enzymes responsible for the oxidation of these amino acids, and the evidence thus obtained indicates that proline and tyrosine are oxidized by specific catalysts. The difference in the ability of animal organs to oxidize the other amino acids suggests the possibility of separate catalysts for some of them.

The purified preparation is made most successfully from rat kidney by a method of adsorption and elution. This preparation by itself takes up no oxygen nor does it reduce methylene blue. It contains very little hemoglobin and only about 30 per cent of the organic matter in the original kidney suspension. With this preparation, it was possible to study quantitatively the oxygen uptake, deamination, and methylene blue reduction of the various amino acids.

## EXPERIMENTAL

A rat is killed by decapitation and the kidneys are chopped with scissors and ground in a mortar with sand after adding 10 cc. of 0.05 M phosphate buffer, pH 7.8. The resulting suspension is pressed through muslin to remove bits of connective tissue. Dilute acetic acid is then added until the pH is just acid to phenol red, *i.e.* 6.9. If the suspension is made more acid the resulting preparation will be inactive. About 0.5 gm. of Celite Analytical

Filter-Aid, a silicate made by Johns-Manville, is now added and the mixture stirred for 5 minutes. Ordinary kaolin can be substituted for the celite but the resulting preparation is not so active. Fullers' earth, Lloyd's reagent, and charcoal were also tried, but without success. Other procedures, such as precipitation by alcohol, acetone, and ammonium sulfate, gave inactive preparations. The mixture is then centrifuged and the liquid layer discarded. 6 cc. of 0.05 M phosphate buffer, pH 7.8, and 1 cc. of 0.1 M sodium carbonate are now mixed with the celite, and allowed to stand for 30 minutes with occasional stirring. The mixture is then centrifuged, and three layers separate out. At the bottom of the centrifuge tube is the celite, then a ring of hemoglobin, and finally a light brown cloudy solution. This is sucked off and constitutes the purified preparation which oxidizes all of the amino acids mentioned above except tyrosine. This preparation still takes up a small amount of oxygen and will reduce methylene blue slowly. It is therefore dialyzed for 1 to 2 hours against running distilled water, after which it shows no oxygen uptake or methylene blue reduction unless a suitable amino acid is added. 1 cc. of the preparation with 1 cc. of buffer, pH 7.8, is used in the Warburg vessels and the Thunberg tubes. Through 1 cm. of the solution the absorption bands of hemoglobin are only just visible with the spectroscope. This preparation will remain active for several days if kept on ice. Comparing it with an untreated broken cell suspension of equal activity, 70 per cent of the organic matter (measured by the difference in the dried weights) has been removed by the purification.

The kidneys of the cat and dog treated in the same way will yield a similar preparation almost as active as that obtained from the rat. If the preparation is made from the livers of these animals, only proline is oxidized by it. This separation demonstrates, therefore, that a catalyst for proline is present in the liver that can be considered different from the catalysts responsible for the oxidation of the other amino acids thus far tested. The liver preparations contain more hemoglobin and are not as pure as the corresponding kidney preparations.

Krebs (4) has shown that rat kidney slices are able to deaminate the natural and unnatural isomers of the amino acids. In the case of alanine, phenylalanine, leucine, and valine the unnatural

isomer was deaminated to a greater extent than the natural isomer. This was confirmed for alanine by Neuenschwander-Lemmer and von Leövey (5). Using the purified preparation of rat kidney, we have found that the *dl* mixtures of phenylalanine, alanine, pro-

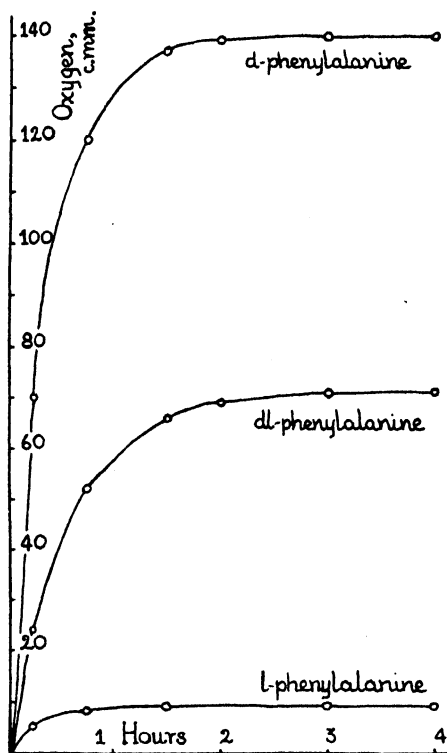


FIG. 1. The oxygen uptake of 2 mg. each of *d*-, *dl*-, and *l*-phenylalanine in the presence of the purified rat kidney preparation, pH 7.8, at 37°. The relationship between the *dl* mixture and the natural (*l*) isomer is typical of the other amino acids.

line, leucine, isoleucine, and valine are rapidly deaminated and oxidized, whereas the natural isomers are attacked slowly or not at all. This apparently means that it is the unnatural isomer of the *dl* mixture that is being attacked. *dl*-Serine and *dl*-methionine were also oxidized and deaminated by the preparation but the

natural isomers have not yet been obtained. Only the natural isomers of tyrosine and oxyproline have been tried. The Eastman Kodak Company supplied the *dl*-leucine, *dl*-isoleucine, and *dl*-methionine, and the rest of the amino acids were supplied by Hoffman-La Roche. Several of the natural isomers were checked with amino acids obtained from casein hydrolysis. The amino acids by themselves neither took up oxygen nor reduced methylene blue. In the presence of the catalyst the average oxygen uptake for each amino acid corresponded to the calculated amount. The nitrogen content by the micro-Kjeldahl method was within 5 per cent of the theoretical, and therefore the amino acids were not purified further. Only one unnatural optical isomer was obtained, namely *d*-phenylalanine, and this, like the *dl* mixture, was rapidly oxidized, whereas the natural *l*-phenylalanine was oxidized very slowly. This is shown in Fig. 1. The results obtained from these experiments with the *dl* mixtures and the natural isomers may thus be considered evidence for determining which isomer is attacked. In using the terms *d* and *l* we have followed Mitchell and Hamilton (6).

The purified kidney extract oxidized all the amino acids tried except tyrosine and oxyproline, the *dl* mixtures of which were not available. This is shown in Table I. The oxygen uptake of the *dl* mixtures was equivalent to the utilization of 1 atom of oxygen per molecule of amino acid oxidized, assuming the oxidation of one isomer. This was true of all the amino acids oxidized by the kidney extract. Some of the natural isomers, for example *l*-proline, were oxidized slowly, others were oxidized hardly at all. The oxygen uptake of the *dl* mixtures should show a rapid oxidation of the unnatural isomer and then a slow oxidation of the natural one. In some cases, for instance with proline and probably methionine, this did happen, but in the majority of cases the oxygen uptake of the *dl* mixtures was equivalent to the oxidation of one isomer. This may possibly be explained by the fact that the oxidation products of the quickly oxidized unnatural isomers inhibit the slow oxidation of the natural ones. On the assumption that all these amino acids except proline are oxidized to the corresponding keto acids, their asymmetry would thus be lost and the oxidation products of both isomers would be the same.

Proline, phenylalanine, and alanine when oxidized by the un-

TABLE I

*Oxygen Uptake and Methylene Blue Reduction of Various Amino Acids in Presence of Purified Rat Kidney Preparation at pH 7.8 and 37°*

1 cc. of the preparation was used with 2 mg. of each amino acid. The preparation alone took up no oxygen, nor did it reduce methylene blue. Column 2 gives the final oxygen uptakes obtained in different experiments selected at random. Column 3 gives the oxygen uptakes calculated on the basis of 1 atom of oxygen for every molecule of amino acid. Column 4 gives the average time in minutes for the oxygen uptake to reach half the theoretical value. Column 5 gives the average time in minutes for the reduction of 0.5 cc. of 1:5000 methylene blue.

Amino acid (1)	Observed O <sub>2</sub> uptake (2)	Calculated O <sub>2</sub> uptake (3)	Oxidation rate (4)	Methylene blue reduction (5)
	<i>c.mm.</i>	<i>c.mm.</i>	<i>min.</i>	<i>min.</i>
<i>d</i> -Phenylalanine	140	136	14	27
	140			
<i>dl</i> -Phenylalanine	74	136	14	29
	71			
	75			
<i>l</i> -Phenylalanine	0	136		91
	9			
<i>dl</i> -Alanine	126	252	15	52
	130			
	123			
<i>d</i> -Alanine	24	252		140
	25			
<i>dl</i> -Proline	126	194	15	19
	120			
	125			
<i>l</i> -Proline	47	194	155	74
	53			
<i>dl</i> -Methionine	85	150	18	31
	89			
	74			
<i>dl</i> -Valine	98	192	21	56
	87			
	102			
<i>d</i> -Valine	14	192		196
	16			
<i>dl</i> -Isoleucine	66*	170	41	41
	84			
	69*			
<i>d</i> -Isoleucine	7	170		182
	5			



TABLE I—*Concluded*

Amino acid (1)	Observed O <sub>2</sub> uptake (2)	Calculated O <sub>2</sub> uptake (3)	Oxidation rate (4)	Methylene blue reduction (5)
	<i>c.mm.</i>	<i>c.mm.</i>	<i>min.</i>	<i>min.</i>
<i>dl</i> -Serine	92 102 56*	214	55	58
<i>dl</i> -Leucine	51* 75* 59*	170	71	25
<i>l</i> -Leucine	6 9	170		81

\* Oxidation not complete.

treated broken cell suspensions were unaffected by 0.005 M KCN (1-3). With the purified kidney extract the oxidation of these and the other amino acids was likewise unaffected by this amount of KCN. The oxidation of serine which was not inhibited at the beginning by KCN was definitely slowed later. This is not a typical KCN effect and is probably due to the direct action of the drug on the enzyme. It has been shown, however, that the oxidation of *l*-tyrosine by untreated liver suspensions is completely inhibited by KCN, whereas the oxidation of certain other amino acids by the same liver suspensions is not. Tyrosine is also the only amino acid the natural isomer of which is rapidly oxidized by liver suspensions although no purified preparation will attack it. The natural *l*-proline is slowly oxidized by the suspensions and purified preparations of livers and kidneys, although less readily than *dl*-proline, while other natural isomers are practically not attacked (see Table I). This differentiates the oxidation of tyrosine and proline from the oxidation of all the other amino acids tried. The inability of KCN to inhibit the oxidation of the majority of these amino acids puts the catalysts responsible for their oxidation in the class with xanthine and tyramine oxidases and shows that they are independent of iron and iron complexes.

In the presence of the purified kidney extract all the amino acids which are oxidized will reduce methylene blue. The rate of reduction of the dye varied with the different amino acids. This is shown in Table I. Moreover, the rate of reduction of methylene

blue is not correlated with the rate of oxygen uptake, as can be seen by comparing Columns 4 and 5 in Table I. Thus leucine was oxidized slowly, yet it reduced methylene blue rapidly, and the opposite was true for valine and alanine. There was also a striking difference in the rate of reduction by leucine and isoleucine. These differences in the reduction time of methylene blue would seem to indicate differences in potential between the various amino acid enzyme systems. In the case of serine, however, the slow reduction is correlated with a slow oxygen uptake. This may mean that an inadequate amount of enzyme is present in the preparation.

The purified kidney extract contained a very small amount of free ammonia. It was therefore possible to follow accurately the ammonia production of the amino acids. Control experiments showed that no appreciable amount of ammonia was lost in the Warburg vessels from a solution at pH 7.8. When the oxidation was finished the ammonia was determined by a vacuum distillation method followed by Nesslerization. The amount of the deamination corresponded in each case to the oxygen uptake, which is further evidence that only one isomer of the *dl* mixture is attacked. All the *dl* mixtures of the amino acids tried were deaminated except proline. This amino acid is also not deaminated when the untreated broken cell suspensions of liver and kidney were used (1). Evidently deamination does not occur in the first stage of the oxidation of proline. The natural isomers of the amino acids were either not deaminated or showed a small deamination corresponding to the amount of oxygen taken up. *l*-Tyrosine when oxidized by the untreated liver suspensions was not deaminated (3).

Fluoride accelerates and increases the oxygen uptake of certain amino acids, for instance proline and alanine, when the untreated broken cell suspensions are used (2). With the purified kidney extract, however, fluoride had no effect on the amount of oxygen taken up by the various amino acids and only a small effect on the rate. This indicates that in the untreated broken cell suspensions the effect of fluoride is not on the first stage of the oxidation of the amino acids but rather on their further oxidation. In the purified extract the oxidation of the amino acids was not carried further, probably because of the absence of the necessary catalysts, and thus the fluoride effect was not observed.

The evidence indicates that tyrosine and proline are oxidized

by specific catalysts. Whether there is a specific catalyst for each amino acid is questionable. By studying the untreated broken cell suspensions of the livers of animals, variations were found suggesting that certain other amino acids do have specific catalysts. For instance, the guinea pig liver suspension will oxidize only proline, tyrosine, and methionine. The tyrosine was inhibited by KCN, though the other two were not, and by making a purified preparation of the liver it was possible to separate the proline from the methionine enzyme. It thus seems that methionine may be oxidized by a separate catalyst. Rat liver oxidized all of the amino acids tried, although more slowly than the kidney. 0.005 M KCN added to the rat liver suspension inhibited only the oxidation of serine. This is probably a direct action of the KCN on the enzyme similar to its effect on the oxidation of serine by the kidney, but it suggests that there may be a specific catalyst for serine. This type of evidence does not demonstrate specificity but suggests the possibility of further separation if the right tissues and animals are used.

In general it may be said that the kidney suspensions of the rat, cat, and dog can oxidize the unnatural isomers of the *dl* mixtures of the amino acids tried. This was also true of the purified kidney preparations of these animals, but the guinea pig kidney preparations were less active and those of the rabbit were inactive. The liver suspensions of the rat, cat, and dog oxidized these *dl* mixtures more slowly than the kidney suspensions. The guinea pig liver suspensions oxidized only *dl*-proline and *dl*-methionine, and the rabbit liver suspensions were inactive. Purified preparations of the livers of these animals, except the rabbit, oxidized only proline.

Although glycine was not oxidized by any liver or kidney suspensions thus far tried, *dl*-phenylglycine was slowly oxidized by the broken kidney cell suspensions of the rat and guinea pig. This slow oxidation may be due to its relative insolubility. The oxygen uptake and deamination again showed that only one isomer was attacked, but which one has not been determined. The purified preparation of rat kidney, however, did not oxidize this compound.

#### DISCUSSION

The use of fairly pure enzyme preparations has made possible a quantitative study of the first stage in the oxidation of various

amino acids. This has been useful in determining the exact amount of oxygen taken up, ammonia liberated, and the relative rates of methylene blue reduction. Evidence of the occurrence of specific catalysts for proline and tyrosine has also been obtained, and there is some indication that such may exist also for methionine and serine. The preferential oxidation of the unnatural optical isomers by the broken cell suspensions and the purified extracts is difficult to explain. Stekol (7) has shown that the sulfur from *dl*-cystine fed to animals is excreted, whereas that of the natural *l*-cystine is retained. This indicates the preferential oxidation of the *d*-cystine. Cox and Berg (8) have shown that the unnatural *d*-histidine can maintain growth in animals almost as well as the natural isomer. It may be that the natural isomers are racemized in the body. If this is not so, then the existence of catalysts which oxidize the unnatural isomers seems explicable only on the basis of chance. Investigations of the end-products of these oxidations are now being carried out.

#### SUMMARY

1. A method is described for obtaining a purified preparation from the kidney of the rat, cat, or dog which oxidizes alanine, phenylalanine, valine, leucine, isoleucine, proline, serine, and methionine.

2. A study of the *dl* mixtures and the natural isomers of the first six of these amino acids indicates that the unnatural isomers are preferentially oxidized. The natural isomers of serine and methionine have not yet been tested.

3. Similar preparations of the livers of these animals oxidize only proline.

4. All the unnatural isomers of the amino acids oxidized by the kidney preparations are deaminated except proline. The amount of the deamination corresponds to the oxygen uptake.

5. 1 atom of oxygen is taken up for every molecule of amino acid oxidized.

6. The oxidation of the amino acids by the kidney preparations is not affected by 0.005 M KCN.

7. Methylene blue is reduced by all these amino acids in the presence of the preparation but at varying rates.

8. *dl*-Phenylglycine is oxidized by the broken cell suspensions

of rat kidney but not by the purified preparation. Glycine is not oxidized by any of the preparations tried.

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## THE IODOMETRIC DETERMINATION OF CYSTEINE\*

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Lucas and King (1) and Virtue and Lewis (2) have recently proposed essentially similar methods for the estimation of cysteine, wherein an excess of iodine is back titrated at 0° in *N* HCl, as being a more satisfactory procedure than that of Okuda (3), since under their conditions the theoretical equation for the oxidation of —SH to —S—S— can be used. However, even at 0° the excess of I<sub>2</sub> present, as well as the time interval before titration, must be limited to as little as possible because of the tendency of the oxidation to proceed beyond the disulfide stage. In the present work it was found that when both the HCl and KI concentrations are made 1 *M*, results are obtained at room temperature which closely approximate the theoretical and which agree over a period of several hours.

The stock solution of KI was 5 *M* and remained colorless when kept at 0° in darkness. In order to minimize the photochemical decomposition of the combined KI and HCl, which will hereafter for convenience be referred to as HI, the solutions were kept either in darkness or at least out of direct light. The following determinations were all conducted at room temperature (25–30°).

Investigation of the behavior of the disulfoxide of *L*-cystine (4) and of the sulfinic acid (5) in such HI solutions showed practically quantitative reduction to cystine when the HI concentration was 1 *M*, as determined by the amount of I<sub>2</sub> formed and by isolation and identification of the cystine (6). That cystine dissolved in a *M* HI solution is stable in the presence of excess I<sub>2</sub> is shown in Table I.

Although the oxidation of cysteine might possibly proceed

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otherwise than through the disulfide stage (*i.e.*— $\text{SH} \rightarrow \text{SOH} \rightarrow \text{SO}_2\text{H} \rightarrow \text{SO}_3\text{H}$ ), the fact that such intermediates as the disulfoxide ( $\text{R}-\text{SO}-\text{SO}-\text{R}$ ) and the sulfinic acid ( $\text{R}-\text{SO}_2\text{H}$ ) are reduced to cystine indicates that oxidation of cysteine by  $\text{I}_2$  in  $\text{M HI}$  would proceed only to cystine.

In the following experiments designed to test the validity of this assumption, the iodine was added to the cysteine (or *vice versa*) in  $\text{HI}$  of such concentration that after dilution to the required volume (usually 50 cc.) the indicated molarity of  $\text{HI}$  was obtained. A blank was also prepared in the same manner. Aliquots of the two solutions (usually 10 cc.) were then titrated with thiosulfate (0.025  $\text{N}$ ), the difference between the two results being due to the  $\text{I}_2$  consumed by the cysteine. In cases of higher acid concentration the solutions were diluted to 1  $\text{N}$  before titration. Since the color of starch-iodine complex changes but slowly in the  $\text{HI}$  solu-

TABLE I  
*Stability of Cystine (0.004 M) against  $\text{I}_2$  in 1 M HI*

Time.....	10 min.	45 min.	105 min.	165 min.	19 hrs.	43 hrs.
$\text{N I}_2 \times 10^3$ in solution.....	20.9	21.3	21.6	21.9	27.3	45.6
" " $\times 10^3$ " blank.....	20.8	21.3	21.6	21.8	27.3	45.2

tion on addition of thiosulfate, the disappearance of the yellow  $\text{I}_2$  color was taken as the end-point and checked (or corrected) in the following manner. 5 to 10 cc. of 0.1 per cent starch solution containing 0.1 per cent salicylic acid were added at the end-point; if the solution was blue (or brownish violet) about 0.02 cc. portions of thiosulfate were added with 10 seconds of shaking between additions until the solution became colorless; when colorless or if already colorless the end-point was verified by adding several drops of dilute iodine equivalent to 0.01 cc. of thiosulfate, whereupon the brownish violet color should return.

In Table II are reported the results obtained on a commercial preparation of cysteine hydrochloride. Variations in  $\text{HCl}$  and  $\text{KI}$ , within the limits shown, are seen to be without effect on the values obtained; the concentration of  $\text{HI}$  adopted as most suitable was 1  $\text{M}$  on the basis of data obtained on the reduction of the intermediate oxidation products mentioned. In  $\text{M HI}$  the changing of

the amount of excess  $I_2$  from about 10 per cent to over 1000 per cent, by varying the cysteine concentration, was also found to be without effect on the results which are moreover seen to be constant over a period of 3 hours.

The fact that the values obtained are independent of the variations in iodine, iodide, and acid concentrations indicates that the correct result was obtained.

TABLE II  
*Iodometric Determination of Cysteine*

	HCl	KI	Cysteine hydrochloride after	
			3-20 min.	1 hr.
	<i>M</i>	<i>M</i>	<i>per cent</i>	<i>per cent</i>
0.04 N I <sub>2</sub> ; 0.028 M cysteine	1.5	1.5	86.4	86.1
	1.5	1.0	86.1	85.6
	1.0	1.0	85.0	84.5
	1.0	0.5	85.9	
	0.5	0.5	86.1	
Average.....			85.7 ± 0.5%	

	Cysteine	Cysteine hydrochloride after	
		30-50 min.	3 hrs.
	<i>M</i>	<i>per cent</i>	<i>per cent</i>
0.04 N I <sub>2</sub> ; 1 M HI	0.0367	85.3	86.0
	0.0261	85.9	85.4
	0.0177	84.0	85.9
	0.0131	85.3	85.5
	0.0089	84.6	84.8
	0.0026	83.3	85.0
Average.....		84.7 ± 0.8	85.4 ± 0.4

The analysis of a second commercial preparation is shown in Table III. The conclusion that the impurity in this preparation is chiefly water is supported by the agreement between the loss in weight on drying<sup>1</sup> and the determinations of HCl and cysteine.

<sup>1</sup> The conditions of drying were established by Dr. K. Shinohara of this Institute.



# 144 Iodometric Determination of Cysteine

A disadvantage in the use of high concentrations of HI is its decomposition to form  $I_2$  (*cf.* Tables I and III), which is influenced by the  $HI_3$  concentration. While no significant change occurs in the cysteine values (the difference between the  $I_2$  content of blank and solution) over a period of about 5 hours, beyond this time

TABLE III  
*Analysis of Cysteine Hydrochloride*

Time ..... | 5-10 min.      1 hr.      2 hrs.      3 hrs.

Experiment 1. 0.06704 gm. R-SH-HCl per 50 cc.; theory  
0.00851 M R-SH

					% theoretical
N $I_2$ blank.....	0.02089	0.02130	0.02144	0.02174	
" " solution.....	0.01299	0.01340	0.01347	0.01380	
M R-SH.....	0.00790	0.00790	0.00797	0.00794	93.1 $\pm$ 0.3

Experiment 2. 0.0644 gm. R-SH-HCl per 50 cc.; theory  
0.00817 M R-SH

N $I_2$ blank.....	0.02090	0.02125		0.02162	
" " solution.....	0.01313	0.01352		0.01389	
M R-SH.....	0.00777	0.00773		0.00773	94.8 $\pm$ 0.3

Experiment 3. 0.1002 gm. R-SH-HCl per 50 cc.; theory =  
0.01272 M R-SH

N $I_2$ blank.....	0.03104	0.03125		0.03158	
" " solution.....	0.01845	0.01873		0.01905	
M R-SH.....	0.01259	0.01252		0.01253	98.6 $\pm$ 0.3

*Experiment 1*—HCl = 93.1 per cent of the theoretical as determined by titration with NaOH, with methyl red as indicator.

*Experiment 2*—Loss on drying for 5 hours at room temperature over  $P_2O_5$ , 1.2 per cent; HCl = 94.4 per cent of the theoretical.

*Experiment 3*—Loss on drying 41 hours (constant weight) at room temperature over  $P_2O_5$ , 6.0 per cent; HCl = 99.4 per cent of the theoretical.

discrepancies appear which are possibly occasioned by different rates of  $I_2$  formation in the solution and blank.

The determination of cysteine, prepared by reduction of cystine with nascent hydrogen, yielded essentially the same results. 0.2402 gm. of cystine in 100 cc. of N HCl (final volume) were reduced

with Sn dust,<sup>2</sup> the dissolved Sn was precipitated by H<sub>2</sub>S, and the H<sub>2</sub>S blown out by CO<sub>2</sub>. The iodometric cysteine determination showed  $101.4 \pm 0.4$  per cent of the theoretical value, and after 48 hours standing,  $99.1 \pm 0.4$  per cent. Reduction of 1.201 gm. of cystine in 50 cc. of 2 N HCl (final volume) with an excess of amalgamated Zn for 17 hours resulted in a solution which gave by the iodometric determination 98.6, 99.8, and 99.7 per cent of the theoretical cysteine content after 0.1, 1, and 2 hours respectively.

It may be concluded from the above results that the determination of cysteine can be carried out under the specified conditions with an error of about  $\pm 0.5$  per cent, which compares favorably not only with that of the existing methods of cysteine estimation but also with the present criteria of purity of cysteine.

#### SUMMARY

Cystine was found to be stable against I<sub>2</sub> when in a solution of m HCl and KI. The indirect iodometric determination of cysteine in m HCl and KI at room temperature was shown to yield results consistent with its oxidation to cystine.

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<sup>2</sup> Unreported data of Dr. Shinohara show the reduction of cystine in HCl solutions to be more satisfactory with Sn dust than with Zn dust.



# **METABOLISM OF *l*- AND *dl*-METHIONINE IN ADULT AND GROWING DOGS MAINTAINED ON DIETS OF VARIOUS PROTEIN CONTENTS**

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In previous communications it has been shown that *l*-cystine sulfur, when fed to adult dogs maintained on a protein-free diet (1) or to pups maintained on low sulfur diets (2), is retained by the animals to a large extent. Comparable amounts of *dl*-cystine sulfur, when fed to adult or growing dogs under the conditions used in experiments with *l*-cystine, were not retained as well as *l*-cystine sulfur. An appreciable portion of *dl*-cystine sulfur appeared in the urine of animals as neutral sulfur.

Inasmuch as recent studies (3-6) have indicated a close physiological relationship between *l*-cystine and *l*- or *d*-methionine, it seemed of interest to compare the metabolism of *l*- and *dl*-methionine to that of *l*- and *dl*-cystine in adult and growing dogs, the same conditions as used in our previous work (1, 2) being employed. Since both isomers of methionine were shown to be capable of promoting growth in rats (3), it was anticipated that both forms of methionine sulfur, in analogy to *l*-cystine sulfur, would be retained by adult dogs fed protein-free diets, and by growing dogs maintained on a low sulfur diet.

## **EXPERIMENTAL**

In order to secure data on the metabolism of *l*- and *dl*-methionine in adult and growing dogs which would be comparable to that procured with *l*- and *dl*-cystine in the same animals, the general experimental procedure used in the present work was the same as the one previously reported (1, 2). The composition of the diets is recorded in Table I. The low protein diet yielded about one-half the casein per kilo of body weight of the pups as did Cowgill's

diet. All pups received in addition 50 gm. of tomato juice and 3 cc. of cod liver oil *per diem* mixed with the food. The protein-free diet yielded 0.16 per cent nitrogen and 0.024 per cent sulfur. The nature of this nitrogen was not determined. Assuming that this nitrogen was entirely that of a protein nature, the diet then contained about 1.0 per cent protein. Inasmuch as experiments with *l*-cystine on adult dogs maintained on a diet of sucrose and fat yielded precisely the same results as those already reported (1), procured on adult dogs maintained on the diet described here as protein-free, we felt justified in using the latter as a protein-free

TABLE I  
*Composition of Diets*

	Cowgill's*	Low protein	Protein-free
Casein, † gm.....	43.7	22.0	0
Sucrose, ".....	40.6	62.3	84.3
Squibb's vitavose, gm.....	11.6	11.6	11.6
Salt mixture, ‡ gm.....	1.4	1.4	1.4
Bone ash, gm.....	2.7	2.7	2.7
Butter fat, gm.....	7.0	7.0	7.0
Lard, gm.....	17.0	17.0	17.0
Nitrogen, per cent.....	4.92	2.57	0.16
Sulfur, per cent.....	0.250	0.125	0.024

\* Cowgill, G. R., Deuel, H. J., Jr., and Smith, H. H., *Am. J. Physiol.*, **73**, 106 (1925).

† Casein No. 453 purchased from The Casein Manufacturing Company of America, Inc.

‡ As used by Karr, W. G., *J. Biol. Chem.*, **44**, 255 (1920).

diet in preference to that of sucrose and fat. Moreover, sucrose and fat diet is not only protein-free but lacks other essential constituents and the animals show undesirable reluctance to eat the food at an early stage of the experiment. The protein-free diet as described here was consumed readily within 10 to 15 minutes. The diet yielded 70 to 80 calories per kilo of body weight. The dogs were kept on this diet for not longer than 30 to 40 days, when they were fed Cowgill's diet for several days. They were then returned to the protein-free diet. Each dog was maintained on the protein-free diet for 2 weeks before the compounds were administered. The compounds were fed mixed with the food which

was fed once a day at 9.15 a.m. The urine was collected every 24 hours at 9 a.m. by catheterization. Water was allowed *ad libitum*. The dogs used in the present work are the same animals as described previously (1).

Pups of 4 to 6 weeks of age were placed in individual metabolism cages and fed Cowgill's diet supplemented by 50 gm. of tomato juice and 3 cc. of cod liver oil *per diem*. In Table V the exact amount and kind of diet fed to the pups at the time of administration of methionine are indicated. The pups were weighed at frequent intervals and the amount of diet was adjusted with each

TABLE II  
*Rate of Gain As Related to Diet Fed*

Pup No.	Age	Diet*	Gain in weight per 100 gm. diet
	<i>days</i>		<i>gm.</i>
5 ♂	48- 88	Cowgill's	50
7 ♀	48- 88	"	37
14 ♂	35- 82	"	49
15 ♀	65- 86	"	65
15 ♀	86-140	Low protein	36
15 ♀	140-170	Cowgill's	69
16 ♂	62-101	"	38
16 ♂	101-131	Low protein	23
16 ♂	131-160	Cowgill's	40

\* The amount of food fed to the pups per kilo of body weight was the same in all cases, yielding 140 calories per kilo of body weight. The intake of food was increased with each approximately 0.5 kilo gain in body weight.

approximately 0.5 kilo increase in body weight, so that the caloric intake, nitrogen, and sulfur per kilo of body weight could be maintained practically constant. Such an adjustment in the amount of food was made approximately every 10 to 12 days on pups maintained on Cowgill's diet. Pups which were fed a low sulfur diet showed a much slower rate of growth and the increase of food intake was not made as frequently as in the case of pups fed Cowgill's diet. No administration of methionine was made immediately after the increase of food; in all cases the pups were allowed to reach a nitrogen and sulfur balance, which was generally attained within 5 to 6 days after the adjustment in food intake. The rate of gain of all pups, calculated on the basis of 100 gm. of diet

consumed, is shown in Table II. Pups 5, 7 (litter mates), and 15 were raised to full maturity on Cowgill's diet. The data on the rate of gain of the pups are presented, covering only the period directly related to the present work. A more complete summary

TABLE III

*Metabolism of dl-Methionine in Growing Dogs Maintained on Cowgill's Diet*

Pup No.	Age	Weight	Urinary output				
			Total N	Urea N	Total S	Total SO <sub>4</sub> S	Neutral S
	<i>days</i>	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
5 ♂	68	4.30	3.45	2.81	0.149	0.124	0.025
	69		3.47	2.89	0.140	0.119	0.021
	70		3.54	2.87	0.197	0.161	0.036*
	71		3.65	2.96	0.159	0.129	0.030
	72		3.58	2.81	0.152	0.117	0.036
7 ♀	72	2.58	2.58	2.08	0.110	0.080	0.030
	73		2.42	1.94	0.089	0.069	0.020
	74		2.60	2.18	0.163	0.130	0.033*
	75		2.52	2.04	0.116	0.084	0.032
	76		2.51	1.97	0.103	0.071	0.032
14 ♂	72	1.95	1.84	1.54	0.118	0.050	0.068
	73		1.70	1.40	0.103	0.048	0.055
	74		2.04	1.66	0.229	0.164	0.065†
	75		1.62	1.37	0.136	0.084	0.052
	76		1.76	1.39	0.121	0.070	0.051
15 ♀	72	3.10	2.50	2.03	0.131	0.070	0.061
	73		2.57	2.15	0.127	0.067	0.060
	74		2.54	2.03	0.209	0.134	0.075†
	75		2.55	2.15	0.150	0.089	0.061
	76		2.51	2.21	0.138	0.078	0.060
16 ♂	76	2.73	2.72	2.41	0.152	0.091	0.061
	77		2.83	2.59	0.155	0.093	0.062
	78		2.91	2.40	0.215	0.154	0.061†
	79		3.02	2.66	0.187	0.127	0.060
	80		2.90	2.59	0.159	0.100	0.059

\* 0.34 gm. of *dl*-methionine was fed with the food; 0.067 gm. of S.

† 0.67 gm. of *dl*-methionine was fed with the food; 0.134 gm. of S.

of the data on the growing dog accumulated so far is reserved for future communications.

All pups were fed twice daily at precisely the same time, 9 a.m. and 12 noon. The compounds were fed at 9 a.m. mixed with the

food. The urine was collected every 24 hours at 9 a.m. and analyzed on the same day. Frequent urination and comparatively large volumes of urine excreted by the pups account, perhaps, for the insignificant variations in the composition of the urine from

TABLE IV

*Metabolism of l-Methionine and dl-Methionine in Growing Dogs Maintained on Low Protein Diet*

Pup No.	Age	Weight	Urinary output				
			Total N	Urea N	Total S	Total SO <sub>2</sub> S	Neutral S
	days	kg.	gm.	gm.	gm.	gm.	gm.
15 ♀	94	4.54	1.96	1.40	0.084	0.033	0.051
	95		2.02	1.54	0.084	0.032	0.052
	96		1.65	1.29	0.113	0.053	0.060*
	97		1.57	1.07	0.104	0.050	0.054
	98		2.16	1.43	0.095	0.038	0.057
	99		2.10	1.56	0.098	0.038	0.060
	132		2.00	1.55	0.090	0.036	0.054
	133		2.08	1.61	0.093	0.037	0.056
	134		1.67	1.24	0.113	0.058	0.055†
	135	5.45	1.60	1.20	0.097	0.047	0.050
	136		2.03	1.57	0.090	0.039	0.051
16 ♂	111		2.00	1.63	0.097	0.040	0.057
	112	3.90	1.96	1.59	0.100	0.042	0.058
	113		1.93	1.55	0.098	0.041	0.057
	114		1.59	1.22	0.111	0.054	0.057*
	115		1.55	1.20	0.110	0.052	0.058
	116		1.97	1.60	0.095	0.039	0.056
	117		2.00	1.66	0.090	0.040	0.050
	118		1.62	1.37	0.110	0.062	0.048†
	119		1.54	1.30	0.099	0.049	0.050
	120	4.18	2.07	1.58	0.091	0.037	0.054
	121		1.99	1.60	0.092	0.041	0.049

\* 0.67 gm. of dl-methionine was fed with the food; 0.134 gm. of S.

† 0.67 gm. of l-methionine was fed with the food; 0.134 gm. of S.

day to day, even though the urine was collected without catheterization. The data recorded in Tables III and IV illustrate the point in question. Water was allowed *ad libitum*.

The plan of the experiments on adult and growing dogs was to feed the diet to dogs or pups for several days and analyze the urine daily until the values of various urinary constituents showed little



or no variation from day to day, to feed the compound mixed with the food, then to continue the experiment until the output of these excretory products had returned to normal.

The following methods of analysis of urine were used: total N, Kjeldahl; urea, Van Slyke's gasometric method (7); inorganic and ethereal sulfates, Folin (8); total sulfur, Denis' modification of Benedict's method (9); creatine and creatinine, Folin's (10); picric acid was recrystallized and purified according to Benedict (11); ammonia, Folin (12).

A portion of the *l*-methionine used in this work was prepared from egg albumin by the method of Pirie (13). The remainder of *l*-methionine was kindly furnished by Dr. N. W. Pirie of Cambridge University. *dl*-Methionine was purchased from the Eastman Kodak Company. The purity of the compounds was checked by analysis.

### Results

The results are presented in Tables III and IV and in summarized form in Table V. In experiments with adult dogs maintained on a protein-free diet, total nitrogen, urea, ammonia, creatine, creatinine, and ethereal sulfate values did not show any significant variations on the day of the feeding of either *l*- or *dl*-methionine. For the sake of economy of space the values for the above constituents were omitted.

*Experiments with l- and dl-Methionine in Adult Dogs Maintained on Protein-Free Diet*—The data in Table V seem to indicate that 63 to 71 per cent of both forms of methionine sulfur are retained by adult dogs maintained on a protein-free diet. In one experiment on Dog 15, feeding of *dl*-methionine produced a small rise in the output of neutral sulfur in the urine. The NaCN-nitroprusside test on the urine was, however, negative. The negative test on the urine, after feeding of methionine, was perhaps due to the fact that much smaller amounts of methionine were administered than reported by other workers (6). All dogs excreted the unutilized methionine sulfur as inorganic sulfate in 2 days. No effects on total nitrogen or any of the constituents which we determined were observed on the day of feeding of *l*- or *dl*-methionine. The results obtained with *l*- and *dl*-methionine feeding to adult dogs maintained on a protein-free diet are similar to those re-

ported on *l*-cystine metabolism in the same dogs kept under similar conditions (1).

*Experiments with dl-Methionine in Growing Dogs Maintained on Cowgill's Diet*—The data in Tables III and V seem to indicate that 74 to 100 per cent of the administered *dl*-methionine sulfur is

TABLE V  
*Metabolism of l- and dl-Methionine in Adult and Growing Dogs*

	Age	Methio- nine fed	Methionine excreted as		Recov- ered	Diet per 24 hrs.	Intake per kilo body weight	
			Inor- ganic S	Neu- tral S			S	N
	days	gm.	per cent	per cent	per cent	gm.	mg.	gm.
Pup 5	70	<i>dl</i> - 0.34	85.0	0	85.0	Cowgill's 112	65	1.30
" 5	74	" 0.34	74.0	0	74.0	" 112	65	1.30
" 7	69	" 0.34	89.0	0	89.0	" 74.4	70	1.40
" 7	73	" 0.34	82.0	0	82.0	" 74.4	70	1.40
" 7	105	" 0.34	100.0	0	100.0	" 93.0	65	1.30
" 14	74	" 0.67	90.0	10.0	100.0	" 56.0	70	1.35
" 15	74	" 0.67	50.0	11.0	61.0	" 88.0	72	1.40
" 15	80	" 0.67	65.0	10.0	75.0	" 88.0	72	1.40
" 16	78	" 0.67	70.0	0	70.0	" 88.0	72	1.40
" 15	96	" 0.67	25.0	0	25.0	Low protein 133.0	34	0.69
" 15	106	" 0.67	20.0	0	20.0	" " 133.0	34	0.69
" 16	114	" 0.67	19.0	0	19.0	" " 111.0	36	0.70
" 15	134	<i>l</i> - 0.67	19.0	0	19.0	" " 154.0	34	0.69
" 16	118	" 0.67	21.0	0	21.0	" " 111.0	36	0.70
Dog 7	645	<i>dl</i> - 0.67	32.0	0	32.0	Protein-free 124.0	4	0.026
" 7	651	" 0.67	37.0	0	37.0	" 124.0	4	0.026
" 12	Adult	" 0.67	37.0	0	37.0	" 136.0	2	0.014
" 15	"	" 0.67	24.0	10.0	34.0	" 124.0	3	0.022
" 15	"	<i>l</i> - 0.67	30.0	0	30.0	" 124.0	3	0.022
" 15	"	" 0.67	29.0	0	29.0	" 124.0	3	0.022

metabolized by the growing dog maintained on Cowgill's diet to yield inorganic sulfates in the urine. The excretion of the extra inorganic sulfur extended over 2 days. In three experiments (Pups 14 and 15) a small fraction of the administered *dl*-methionine sulfur appeared in the urine on the 1st day as neutral sulfur. The NaCN-nitroprusside test on the urine was again negative.

No effects on total nitrogen or any of the constituents which we determined were observed on the day of feeding of *dl*-methionine, or on the days following, to pups maintained on Cowgill's diet. The results with *dl*-methionine are similar to those obtained with *l*-cystine feeding to pups maintained on Cowgill's diet (2).

*Experiments with l- and dl-Methionine in Growing Dogs Maintained on Low Sulfur Diet*—The data in Table IV seem to indicate that both forms of methionine are almost completely and equally well retained by the growing dog maintained on a low sulfur diet. The diet yielded about one-half the amount of sulfur per kilo of body weight of the pup as yielded by Cowgill's diet. 75 to 81 per cent of the ingested methionine sulfur was retained. The remainder was excreted in the urine as inorganic sulfate in 2 days. Both pups showed an unmistakably greater retention of nitrogen on the day of administration of either *l*- or *dl*-methionine and on the day following, as indicated by a drop in the output of total nitrogen in the urine for 2 successive days. The drop in nitrogen was accounted for by a corresponding drop in the output of urinary urea. In both pups fecal nitrogen remained constant throughout the experiments. These findings are comparable to those of Lewis (14) who fed *l*-cystine to adult dogs maintained on low protein diets and to ours (2) on one of the pups which was fed *l*-cystine while maintained on a low protein diet.

#### DISCUSSION

The results obtained with *l*- and *dl*-methionine on adult dogs maintained on a protein-free diet are analogous to those secured with *l*-cystine on the same animals fed a similar diet (1). The data apparently indicate that *l*- and *dl*-methionine sulfur are as readily retained by the dog as *l*-cystine sulfur. As in the experiments with *l*-cystine, the retention of the administered methionine sulfur was not followed by a decreased output of urinary nitrogen. This observation seems to suggest that the retained *l*-cystine or either form of methionine sulfur is utilized by the dog maintained on a protein-free diet, not necessarily in the form of protein, and that the adult dog possesses a store of sulfur other than, and in addition to, the tissue protein. The results seem also to corroborate the observations of previous workers made on pigs (15). These investigators have found that feeding of *l*-cystine to pigs

which were reduced to a minimum endogenous nitrogen balance does not improve appreciably the negative nitrogen balance. Most of the *l*-cystine sulfur fed was, however, retained by the pig, thus inducing a positive sulfur balance. Lewis (14) has postulated that feeding of *l*-cystine to adult dogs maintained on low protein diets improves the nitrogen balance of the dog. Our results seem to suggest that the observation of Lewis (14) does not apply to dogs maintained on a protein-free diet, and that the output of endogenous nitrogen in the urine is independent of the food sulfur.

Growing dogs retained *l*- and *dl*-methionine sulfur while maintained on a low protein diet. The diet yielded half as much sulfur per kilo of body weight of the pups as Cowgill's diet. On Cowgill's diet, no retention of *dl*-methionine sulfur took place. The data recorded in Table II indicate that the pups showed a marked retardation in the rate of gain when they were given the low protein diet. We preferred to use the same animal in comparing the nutritional value of our diets rather than to make comparisons of different animals. This procedure seems more reliable, particularly because our animals were mongrels and presented considerable individual variations in the rate of gain, even when maintained on the same diet. The results seem to indicate that Cowgill's diet, when fed in the amounts shown in Table V, was adequate in its casein and sulfur content to meet the requirements for growth in dogs. The data on *l*- and *dl*-methionine are comparable to those procured with *l*-cystine on growing dogs (2). The retention of *l*- or *dl*-methionine sulfur by the growing dog was invariably followed by an increase in the utilization of food nitrogen. This observation corroborates our previous findings on one of the pups which was fed *l*-cystine while maintained on a low protein diet (2) and the earlier one of Lewis (14) on adult dogs which were maintained on low protein diets. From the work of Lewis (14) and the results reported here, it seems that in the absence of adequate amounts of sulfur in the diet, a certain portion of the utilizable nitrogen of the food cannot be retained by the animal and is excreted in the urine, mainly as urea. The so wasted nitrogen is apparently that of the metabolized amino acids which under normal dietary conditions would have been retained by the growing dog to build new protein and by the adult dog to replace tissue waste. This is indicated by the observation that the diminished

output of nitrogen in the urine of growing dogs on the day of administration of either *l*-cystine or *l*- or *dl*-methionine is accounted for by a corresponding drop in the output of urinary urea. The depression in the output of urinary nitrogen on the day of administration of *l*-cystine is not apparently due to the sparing action of *l*-cystine on the tissue of adult animals maintained on low protein diets (14). It appears probable that the utilization of cystine or methionine sulfur by the growing dog may perhaps be an indication of the increased synthesis of protein from the administered cystine or methionine and the amino acids of the food which, without adequate amounts of cystine or methionine in the food, would have been metabolized and excreted in the urine as urea. The observations that both forms of methionine (3), in contrast to the results obtained with *d*-cystine (16), promote growth in rats, and that the cystine-deficient rats store less tissue protein than their litter mates receiving *l*-cystine in addition to the cystine-deficient diet (17), seem to corroborate the interpretation of our results.

I wish to express my gratitude to Dr. N. W. Pirie for his generosity in placing at my disposal a sample of *l*-methionine.

#### SUMMARY

1. *l*- and *dl*-methionine were fed to adult dogs maintained on a protein-free diet and to growing dogs maintained on Cowgill's and low sulfur diets.

2. *l*- and *dl*-methionine sulfur were almost completely and equally well retained by adult dogs maintained on a protein-free diet and by growing dogs maintained on low sulfur diets. Retention of *l*- or *dl*-methionine sulfur by the growing dog was accompanied by an increased utilization of food nitrogen.

3. The metabolism of *l*- and *dl*-methionine in adult and growing dogs is similar to that of *l*-cystine in the same animals fed and kept under identical conditions.

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# THE LACTASE ACTIVITY OF THE INTESTINAL MUCOSA OF THE DOG AND SOME CHARACTERISTICS OF INTESTINAL LACTASE

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The early work on intestinal lactase has been reviewed by Plimmer (1906) and Oppenheimer (1925). No uniformity of opinion was reached regarding the presence and distribution of this enzyme in the intestinal mucosa or in intestinal juice. Without attempting to review these investigations again, it suffices to state that some authors found lactase in the small intestine of young animals. Others reported its presence in the intestine of adult animals as well.

It is well known that animals do not utilize lactose readily when it is administered parenterally. When this sugar is given by mouth, however, it is absorbed and utilized. Unless the quantity fed is large, it is not excreted in the urine to any extent. Thus, all evidence points to the probability that lactose given *per os* is normally hydrolyzed before it is absorbed (Corley, 1927). Heretofore, attempts to account for lactose absorption in terms of the observed lactase activity have not been successful. Röhman and Nagano (1903) in their extensive study of sugar absorption in dogs found no lactase activity in intestinal juice and only slight activity in the mucosa. In the Thiry loops of their dogs the observed lactase activity was totally inadequate for the hydrolysis of the lactose absorbed from the loops. They concluded that this sugar was not hydrolyzed prior to its absorption as were sucrose and maltose. Cajori (1933) found that lactose was absorbed from intestinal loops of dogs much more rapidly than could have been predicted from the enzyme activity of the intestinal juice.

The analytical methods available to the early investigators were



not satisfactory. Probably much of the uncertainty about intestinal lactase is ascribable to the analytical difficulties encountered in determining small quantities of glucose and galactose in the presence of larger amounts of lactose. The recent method of Tauber and Kleiner (1932-33) for the determination of monosaccharides has proved to be an adequate tool for quantitative lactase studies. Employing this method, we have reinvestigated the distribution of lactase in the adult dog's small intestine. The results which have been obtained are pertinent to discussions of the nutritive value of lactose (Koehler and Allen, 1934), for we have found lactase in quantities sufficient to account for the hydrolysis of considerable amounts of lactose in the small intestine.

Few studies have been published in which the characteristics of intestinal lactase have been investigated. Added information about this enzyme and the conditions influencing its action is presented in this paper.

#### EXPERIMENTAL

*Enzyme Sources*—Intestinal mucosa, stripped from the duodenum or jejunum of adult dogs, when employed directly without extraction, was prepared for use by grinding weighed samples of the fresh tissue with sand.

Water extracts were also used. They were prepared by extracting mucosa in the cold with an equal weight of toluene water for several days. After filtering through cotton or centrifuging, a turbid, milky extract was obtained which was used without further treatment, except in experiments involving adsorbing agents. These crude extracts kept well at 3° with only moderate decreases in enzyme activity during several weeks.

Intestinal juice was obtained from dogs with Thiry loops<sup>1</sup> and was centrifuged before use.

Either water extracts of fresh dog liver were used or extracts of liver after dehydration and defatting with acetone and ether.

*Enzyme Activity*—The digestion mixtures usually consisted of 1 volume of 5 per cent (0.14 M) lactose solution, 1 volume of 0.2 M buffer solution, acetic acid-sodium acetate at pH 5.6, and 0.5 vol-

<sup>1</sup> For the collection of this juice, Dr. Ravdin and Dr. Johnston of the Department of Research Surgery kindly permitted us to use their fistula dogs.

ume of enzyme solution or the weighed tissue. For monosaccharide analysis, 5 cc. portions were removed immediately after addition of the enzyme and at intervals afterwards. The mixtures, preserved with toluene, were kept at 38°, usually for digestion periods of 1 to 4 hours.

Enzyme solutions, inactivated by heat, were employed as controls in the usual way. No increase in monosaccharides was noted in these control experiments. In some trials with fresh tissue and with liver extracts, substitution of water for the lactose solution revealed an increase of monosaccharides, or substances oxidized by copper acetate. In these cases suitable correction was applied to the lactose results. The formation of reducing substances in intestinal extracts or juice was not observed.

*Monosaccharide Analysis*—In preparing a filtrate for the sugar determination, colloidal iron was found convenient. 1 cc. of colloidal iron (Merck, 5 per cent) and a drop of 20 per cent  $\text{Na}_2\text{SO}_4$  were added to the 5 cc. sample removed from the digestion mixture. The precipitated ferric hydroxide was first separated by centrifuging and filtering and then repeatedly washed with water. The enzyme activity was removed, but not destroyed, by the ferric hydroxide and it was found important to avoid delays in separating the solution and the precipitate. The filtrate was made up to a volume of 50 or 100 cc. and 2 cc. aliquots were used for the monosaccharide determination, according to the directions of Tauber and Kleiner.

The electrolytes, acetates and phosphates, which were used as buffers in the digestion mixtures did not affect the copper reagent in these dilutions. At greater concentrations, phosphates interfered seriously with the Barfoed reagent.

With this method very little color was developed from the lactose present. For this reason it proved especially valuable when enzyme action was weak.

### Results

*Distribution of Intestinal Lactase*—Typical results, obtained when lactose was digested with intestinal mucosal tissue and succus entericus, are given in Table I. It will be noted that the maximum lactase activity was obtained when fresh, finely ground tissue was used. Jejunal mucosa revealed a 10 to 30 per cent greater lactase

activity than duodenal tissue. In the four experiments in which duodenal and jejunal mucosa are compared, the two tissues, after removal from the animal, were subjected to identical treatment before and during the digestion trials. Water extracts of mucosa uniformly showed less enzyme activity than the tissue. Feeble enzyme activity was found in jejunal juice, the range of activity of six juices tested being 0.5 to 3.1 mg. of lactose hydrolyzed per cc. of juice per hour. Juice from a Thiry loop of the colon failed to induce lactose cleavage.

TABLE I

*Lactose Hydrolysis with Dog Intestinal Mucosal Tissue, Mucosal Extracts, and Succus Entericus*

Experiment No.	Enzyme source	Lactose hydrolyzed per hr. per gm. tissue	Experiment No.	Enzyme source	Lactose hydrolyzed per hr. per gm. tissue or per cc. juice
		mg.			mg.
	Fresh mucosal tissue			Mucosal extracts	
1	Duodenal	12.1	10	Duodenal	4.0
	Jejunal	13.8	11	"	4.6
2	Duodenal	9.1	12	"	3.8
	Jejunal	13.2	13	Duodenal-jejunal	5.2
3	Duodenal	32.1	14	"	9.6
	Jejunal	40.2	15	"	4.2
4	Duodenal	14.7	16	"	5.4
	Jejunal	24.2			
5	Duodenal-jejunal	5.7			
6	"	14.6	17	Jejunal juice	1.2
7	"	18.9	18	" "	2.2
8	"	8.4	19	" "	3.1
9	"	26.7	20	" "	0.5

The observation that water extracts of mucosa are less active than the unextracted tissue and that the succus entericus exhibits only slight lactase activity suggests that this enzyme is intimately associated with mucosal cells. This conclusion that the succus entericus is a digestive fluid of only minor importance was recently reaffirmed for a number of enzymes of the small intestine by Cajori (1933). The major digestive action, resulting from enzymes elaborated in the small intestine, would then occur intracellularly or in direct contact with the mucosa.

The lactase activity here reported is less than the maltase and sucrase activity of the intestinal tissue of dogs found by Röhman and Nagano (1903). It is important to determine whether the observed lactase is sufficient to account for the hydrolysis of lactose when this sugar is absorbed from the gut. Cajori (1933) observed in the dog an average absorption of 435 mg. of lactose in 1 hour from a 22 cm. Thiry loop of the jejunum. The area of this loop was estimated to be 88 sq.cm. and to contain 12.5 gm. of mucosa. This latter estimate was based on an average yield of 0.14 gm. of mucosa per sq.cm. of gut. If the 435 gm. of absorbed lactose were hydrolyzed, each gm. of mucosa hydrolyzed 35 mg. of lactose. This is probably a maximal value, for it is quite likely that under the extreme conditions of this experiment some lactose disappeared from the loop unhydrolyzed. The loop is flooded with sugar solution, a condition which must rarely occur during ordinary feeding. It will be observed in Table I, nevertheless, that an occasional active tissue preparation exhibited a lactase activity of this order. This seems significant, for the actual lactase activity of tissue functioning *in situ* is undoubtedly greater than can be demonstrated experimentally. Conditions during testing of enzyme activity must only approximate those existing during digestion and absorption.

In view of these findings one is not forced to conclude, as Röhman and Nagano did, that lactose is necessarily absorbed without being hydrolyzed. However, in this connection it is of some interest to record the finding of slight lactase activity in liver tissue and in water extracts of liver. Although liver rarely hydrolyzed more than 1 mg. of lactose per hour per gm. of tissue, the finding of a liver lactase may be of significance in revealing a further mechanism for lactose hydrolysis in the body.

*Characteristics of Intestinal Lactase*—Water extracts of intestinal mucosa were used in studying the characteristics of lactase and the factors influencing its action.

*pH Optimum*—In determining the pH optimum, 0.2 M acetate or phosphate buffers were used. In the critical range, the pH of the buffers and certain of the digestion mixtures was determined with the glass electrode.<sup>2</sup>

<sup>2</sup> We are indebted to Dr. E. J. de Beer for the measurements with the glass electrode.

Dog intestinal lactase exhibited optimum activity (Chart 1) in solutions of pH 5.4 to 6.0. This optimum pH is similar to that found for intestinal lactase in other animals, but is distinctly different from plant lactases. Freudenberg and Hoffman (1922) state that calf intestinal lactase had an optimum activity at about pH 5.0. Wigglesworth (1927) reported a pH optimum range of 5.0 to 6.4 for gut lactase of the cockroach. Willstätter and Oppenheimer (1922) found that yeast lactase had a pH optimum at 7.0, whereas pH 4.2 was optimum for almond lactase according to Willstätter and Csányi (1921).

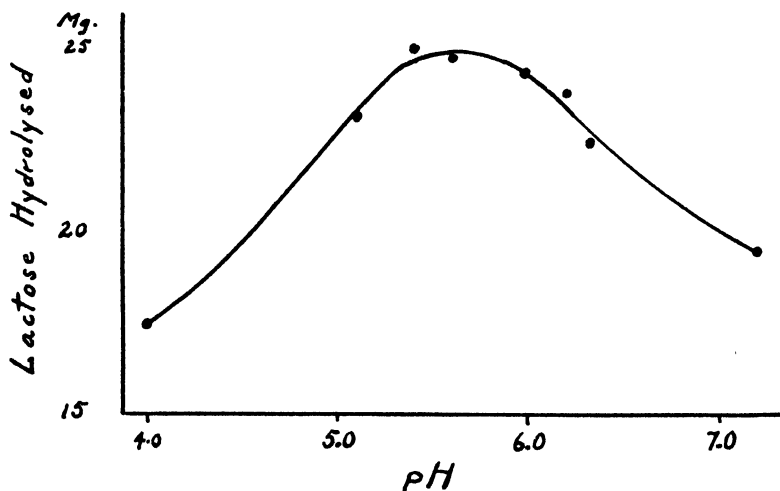


CHART 1. Activity of lactase from dog intestine

*Reaction Course*—Armstrong (1904) studying the hydrolysis of lactose with lactase from kephir-grains observed that the monomolecular reaction constant increased at first, and decreased later. The same thing was found in the case of yeast lactase by Willstätter and Oppenheimer (1922). In our experiments with intestinal lactase, we followed the hydrolysis of lactose at intervals during a 24 hour period. It is interesting to note (Table II) that a similar monomolecular reaction course was observed.

*Effect of Substrate Concentration*—The initial velocity of lactose hydrolysis was found to decrease when the lactose concentration

in the digestion mixtures was less than 2 per cent (0.056 M). At a lactose concentration of 0.006 M the initial velocity was about one-half of the maximum observed with higher lactose concentrations.

Analysis of the data by graphical methods indicated that this value, 0.006 M, may be regarded as a true Michaelis constant and that the mechanism of lactase action involves the combination of 1 molecule of lactose and 1 molecule of enzyme. For evaluation of the dissociation constant,  $K_s$ , in the simplest case of enzyme-substrate combination,  $S + E \rightleftharpoons SE$ , Lineweaver and Burk (1934)

TABLE II

*Lactose Hydrolysis by Intestinal Lactase*

15 cc. of 0.2 M acetate, pH 5.6; 15 cc. of 0.14 M lactose; 7.5 cc. of duodenal-jejunal extract; 1.5 cc. of toluene;  $T = 38^\circ$ . 5 cc. portions removed for monosaccharide analysis.

$t$	Monosaccharides as glucose	$x$ (lactose hydrolyzed)	$K^*$
hrs.	mg.	per cent	
1.0	30	4.0	0.0177
2.0	62	8.3	0.0188
3.0	92	12.3	0.0190
5.0	146	19.5	0.0188
7.0	191	25.5	0.0183
23.5	469	62.5	0.0181

\*  $K = (1/t) \log (100/(100 - x))$ .

suggest the use of the linear form of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_s}{V_{\max} S} + \frac{1}{V_{\max}}$$

When the reciprocals of the velocities ( $v$ ) were plotted against the reciprocals of the lactose concentrations ( $S$ ) a straight line resulted. Following Lineweaver and Burk's method, the slope of the line  $K_s/V_{\max}$  was determined and  $V_{\max}$  obtained by straight line extrapolation. In Experiment 1,  $K_s$  was calculated as 0.0055 and in Experiment 2 as 0.0061. The initial velocities at different lactose concentrations and the initial velocities calculated from the  $K_s$  values 0.0055 and 0.0061 are given in Table III. The agreement

between the observed and calculated velocities justifies the extension of the Michaelis concept to this enzyme. This value for the Michaelis constant is very much lower than the  $K_s$  for gut invertase (Cajori, 1930).

In the presence of glucose, lactase action was retarded, the decrease in lactose hydrolyzed in 22 hours amounting to 40 per cent. On the other hand, galactose in a similar period had practically no effect on lactase activity. In both experiments the initial molar

TABLE III

*Initial Velocity of Lactose Hydrolysis at Different Lactose Concentrations*

	Lactose concentration	Lactose hydrolyzed in 4 hrs.	Relative initial velocity Observed	Initial velocity Calculated $K_s = 0.0055$
	<i>M</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
Experiment 1	0.110	24.3	100	
	0.056	24.4	100	
	0.028	22.5	92	84
	0.014	17.5	72	72
	0.009	15.0	62	62
	0.007	14.4	59	56
	0.0035	9.3	38	39
	0.002	6.0	25	27
Experiment 2				$K_s = 0.0061$
	0.056	30.5	100	
	0.014	23.1	76	70
	0.006	15.0	49	49
	0.0044	11.9	39	42
	0.0017	5.0*	16	22

\* Not used in the calculation of  $K_s$ .

concentration of monosaccharides was twice that of lactose. This result confirms the earlier observations of Stevenson (1912) who characterized gut lactase as glucolactase.

Freudenberg and Hoffman (1922) reported that phosphates accelerated the action of calf intestinal lactase. With dog mucosal extracts, we have been unable to observe that the addition of phosphate had any effect on the rate of lactose hydrolysis. At the same pH, identical amounts of lactose were hydrolyzed with acetate alone and with the acetate plus phosphate.

*Adsorption*—A few experiments are presented recording the behavior of intestinal lactase in the presence of adsorbing agents.

When shaken for a few minutes with kaolin, extracts of intestinal mucosa, acidified with acetic acid, were considerably clarified. There was no loss of lactase activity. In alkaline solutions, kaolin removed considerable amounts of the enzyme. Treatment with kaolin served as a convenient method for partial purification of the crude mucosa extracts. The extracts, containing acetic acid, retained their activity although lactase was found to be very sensitive to strong acid. An extract made acid to Congo red with HCl was completely inactivated in a few minutes.

Ferric hydroxide or alumina cream completely adsorbed lactase from crude or clarified extracts, acidified with acetic acid.

Elution of the enzyme from these adsorbing agents was attempted with dilute NaOH,  $\text{NH}_4\text{OH}$ , or  $\text{Na}_2\text{HPO}_4$  solutions. Elution was incomplete or accomplished with great loss of activity. Maximum recoveries of 20 to 25 per cent of the original lactase activity were obtained with 0.2 M  $\text{Na}_2\text{HPO}_4$ .

Some peptization of ferric hydroxide occurred during elution from this adsorbent and yellow solutions resulted. This was observed with freshly precipitated  $\text{Fe}(\text{OH})_3$  and with preparations that had been aged for a year.

The enzyme solutions obtained after the use of kaolin, adsorption, and elution gave positive biuret tests and contained heat-coagulable protein.

#### SUMMARY

A lactose-splitting enzyme has been found in the duodenal and jejunal mucosa of the dog, in water extracts of the mucosa and, to a lesser extent, in jejunal juice and in liver. The question of lactose hydrolysis in the intestinal mucosa in relation to lactose absorption has been discussed.

Intestinal lactase exhibited maximum activity in acid solution, between pH 5.4 and 6.0. Its activity was inhibited by glucose but not by galactose. The presence of phosphates had no effect on the lactase activity. A determination of the Michaelis constant revealed a  $K_s$  value of 0.006.

Lactase was found to be adsorbed readily, from slightly acid solution, by aluminum hydroxide or ferric hydroxide.



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## THE ENZYMATIC HYDROLYSIS OF RAW AND HEAT-TREATED EGG WHITE

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The effect of a preliminary heat treatment of proteins upon their digestion *in vitro* appears to be dependent to some degree on the nature of the protein. The studies of Waterman and Johns (1) and of Jones and Waterman (2) have demonstrated that a partial cooking of phaseolin, casein, and cottonseed globulin increases the extent of hydrolysis of these proteins by the successive action of pepsin and trypsin. The digestion of arachin, however, was uninfluenced by heat treatment prior to enzymatic action.

The question of the relative physiological value of raw and of heat-treated egg white has served to emphasize again the importance of heat as a factor in determining the extent of utilization of proteins. Investigations dealing with this subject have in the majority of instances involved growth or nitrogen balance studies; conflicting results have been reported (3-7). Of particular interest are the recent studies of Parsons and coworkers (8-10) and of Salmon and Goodman (11) on the nutritional disorder produced by feeding large quantities of raw egg white to rats.

In view of the conflicting results and diverse explanations concerning egg white in nutrition, an investigation of the *in vitro* hydrolysis of raw and of heat-treated egg white has been conducted. Data have been obtained concerning: (1) the comparative rates of digestion of raw and of heat-treated egg white *in vitro*, and (2) the extent of hydrolysis of both types of substrate by pepsin and trypsin acting both individually and successively.

### EXPERIMENTAL

A stock egg white powder was prepared by drying the whites of fresh eggs *in vacuo* over sulfuric acid at 25°; the dried product

was ground to a fine powder and thoroughly mixed. Standard egg white solutions were prepared by suspending 5 gm. of the powder in 500 cc. of water, shaking until a uniform suspension was obtained, and then slowly adding with shaking 450 cc. of boiling distilled water. The mixture was thoroughly agitated until solution was practically complete,<sup>1</sup> cooled to room temperature, and diluted to 1000 cc. with distilled water. 100 cc. aliquots were withdrawn; for purposes of reference, these will be called Samples A, B, C, D, etc.

Aliquot Sample A was adjusted to pH 8.0 with 1 per cent sodium hydroxide, warmed on a water bath to 40°, and treated with 10 cc. of a 1 per cent trypsin<sup>2</sup> solution. The flask was shaken thoroughly and a 10 cc. portion withdrawn for analysis. To the remainder of the solution were added 5 cc. of a 0.4 per cent thymol solution in ethyl alcohol and the flask was stoppered and placed in an incubator maintained at 38°. Samples were withdrawn at varying intervals of time for analysis. Enzymatic action in each withdrawn aliquot was terminated by heating the solution for 5 minutes at 80°. The sample was then allowed to cool to room temperature and titrated by means of the Sørensen formol titration (12).<sup>3</sup>

A second 100 cc. aliquot (Sample B) of the egg white solution was immersed in a boiling water bath for exactly 1½ minutes.<sup>4</sup> In a similar manner, subsequent aliquots (Samples C, D, E, etc.) were heated for 5, 10, 30, 45, 60, and 120 minutes respectively. Each of these solutions was then made up to the original 100 cc.,

<sup>1</sup> A small, barely perceptible amount of material failed to dissolve.

<sup>2</sup> 1:110 trypsin, Difco Laboratories.

<sup>3</sup> A series of check determinations has been conducted in which the increase in carboxyl groups was determined by titration in 90 per cent alcohol according to the method of Willstätter and Waldschmidt-Leitz (13), with thymolphthalein as the indicator. The trend of the values given by the latter procedure was similar to that yielded by the Sørensen method. Since the results in these experiments are of interest because of their relative, rather than their absolute magnitude, we are reporting the titration values obtained with the Sørensen technique. The significance of the measurements by each of these two volumetric methods, and the adequate accuracy of the Sørensen procedure for the present type of investigation, have recently received excellent discussion by Richardson (14).

<sup>4</sup> The egg white solution became slightly opalescent when subjected to the heat treatment, but there was no flocculation of protein.

replacing the water lost by evaporation during the boiling process. These aliquots were then treated with trypsin, incubated, and analyzed at intervals, as described above. For each 100 cc. aliquot, simultaneous control experiments were performed by incubation of (1) 0.1 gm. of trypsin dissolved in 100 cc. of water and (2) 100 cc. of stock egg white solution; both controls were adjusted to pH 8.0, and analyzed at the same intervals as were the withdrawn samples of the experimental digest. The sum of the two titration values from these control solutions was considered as a blank and subtracted from the figure obtained by analysis of the digest aliquot. Table I presents the experimental values of these trypsin studies.

TABLE I  
*Hydrolysis of Egg White by Trypsin*

Each value represents the corrected cc. of 0.05 N sodium hydroxide required to titrate a 10 cc. portion of the digest.

Preliminary heating period	Incubation time, in min.							
	20	40	60	90	120	180	240	300
<i>min.</i>								
0	0.05	0.05	0.05	0.09	0.11	0.11	0.15	0.17
1.5	0.14	0.26	0.28	0.36	0.44	0.52	0.58	0.68
5	0.28	0.50	0.56	0.72	0.82	0.95	1.10	1.15
30	0.48	0.62	0.80	0.82	0.95	1.06	1.12	1.22

Experiments were also conducted in which the action of trypsin was preceded by an incubation period with pepsin. In these experiments, 100 cc. aliquots (Samples A, B, C, etc.) of a stock solution of 0.5 per cent egg white powder in 0.3 per cent hydrochloric acid were used. Sample A was treated with 10 cc. of a 1 per cent solution of pepsin,<sup>5</sup> preserved with thymol, and incubated at 38° for 90 minutes. At the end of this period, a 10 cc. aliquot was withdrawn for analysis; the remaining contents of the flask were adjusted to pH 8.0 with N sodium hydroxide and treated with trypsin, incubated, and analyzed at intervals as already described. Simultaneous blank determinations were conducted on egg white in 0.3 per cent hydrochloric acid and on the enzyme solutions.

<sup>5</sup> 1:3000 pepsin, Parke, Davis and Company.

The experimental values obtained were corrected by these blank titration values.

Aliquot Samples B, C, D, etc., were subjected to preliminary heating periods of varying length, before being treated with pepsin. The subsequent procedure was identical with that outlined in the preceding paragraph. The results of the studies in which trypsin was preceded by pepsin are presented in Table II.

Under the experimental conditions employed, the cleavage of raw egg white by pepsin is negligible, while trypsin exhibits a

TABLE II

*Hydrolysis of Egg White by Trypsin Following a 90 Minute Incubation Period with Pepsin*

Each value represents the corrected cc. of 0.05 N sodium hydroxide required to titrate a 10 cc. portion of the digest.\*

Preliminary heating period	Incubation time, in min.							
	20	40	60	90	120	180	240	300
min.								
0	0.20	0.43	0.55	0.74	0.77	1.00	1.06	1.06
1.5	0.62	0.81	0.85	0.93	1.22	1.32	1.32	1.42
5.0	1.08	1.12	1.26	1.27	1.40	1.42	1.54	1.58
30	1.13	1.19	1.26	1.38	1.49	1.54	1.58	1.71
45	1.34	1.54	1.68	1.80	1.80	2.02	2.08	2.12

\* The values have been obtained by subtraction of the titration figure found at the end of the 90 minute incubation period for pepsin from the titration value resulting after tryptic incubation. These pepsin values, expressed as cc. of 0.05 N sodium hydroxide required to titrate 10 cc. of the digest, were 0.0 cc. for raw egg white, and 0.28, 0.42, 0.70, and 1.50 cc. for egg white subjected to preliminary heating periods of 1.5, 5, 30, and 45 minutes respectively.

limited ability to hydrolyze this substrate.<sup>6</sup> However, despite the apparent inactivity of pepsin with the raw egg white, it appears that this enzyme did cause some change in this material, since tryptic action was much more extensive when preceded by pepsin than when trypsin alone was used (compare Table I and Table II, raw egg white).

A preliminary heating of the egg white solution prior to incuba-

<sup>6</sup> The titration results obtained after peptic action under the conditions described have also been checked by the Northrop technique (15).

tion with the enzymes resulted in a marked increase in the extent of hydrolysis of the substrate. When trypsin alone was used, the degree of hydrolysis of egg white increased as the preliminary heating period was extended to 30 minutes. Longer heating periods did not further the hydrolytic process; the titration data for egg white solutions heated for 30, 45, 60, or 120 minutes previous to incubation with trypsin are identical within the limits of experimental error. When the action of trypsin was preceded by pepsin, there was a continued increase in the magnitude of hydrolysis of egg white as the preliminary cooking period was lengthened to 45 minutes. Egg white solutions heated for 45, 60, or 120 minutes previous to peptic plus tryptic action yield similar data.

#### DISCUSSION

The results which have been obtained are in harmony with those of *in vivo* experiments which have indicated a better utilization of heat-treated than of raw egg white. Of the hypotheses which have been offered to explain this difference in physiological availability, the suggestion that the indigestibility of raw egg white is due to a heat-labile, antitryptic factor appears to be in concordance with the present findings. The recent work of Balls and Swenson (16) on the antitrypsin of egg white is of considerable interest in this connection. These investigators reported the preparation from egg white of a concentrate which inhibits the proteolytic activity of trypsin; the product was described as moderately heat-stable and destroyed by continued boiling. It appears quite possible that under the experimental conditions herein employed the shorter boiling intervals brought about a partial inhibition of the antitrypsin of egg white, while the longer periods of cooking effected a complete destruction of this antienzyme. It is likely, however, that the reported poor utilization and evident toxicity of raw egg white is not due solely to an antitrypsin. The physical characteristics of the material *per se* may be a contributing factor.<sup>7</sup> It seems significant that the lack of nutritional value of the uncooked material may be related to the limited time which it remains in the stomach (17-19). This shorter period would tend to restrict the action of pepsin; the important rôle of this gastric

<sup>7</sup> For an excellent discussion of possible factors involved in the utilization of raw egg white, see reference (3).

enzyme in the proteolysis of egg white is indicated by the results reported in this communication. In fact, a preliminary treatment with pepsin compares favorably with a 30 minute period of cooking of the egg white in its ability to augment the action of trypsin (compare Table I, 30 minute preliminary heating period, with Table II, no preliminary heating period).

The recent report by Calvery (20) of the extensive action of pepsin on crystalline egg albumin is of interest here in view of the conclusion by Bateman (3) that of the individual proteins constituting egg white, the albumin fraction appears to be the indigestible component. These results differ from those described in this communication. However, the substrate utilized by Calvery was crystalline egg albumin; the preparation of this material in a highly purified state might readily eliminate any enzyme-inhibiting factor present in the native egg white. Furthermore, in the experiments of Calvery 2.5 gm. of enzyme were employed for 1 gm. of egg albumin, whereas in the present investigation only 0.2 gm. of the proteolytic agent was used for each gm. of substrate.

#### SUMMARY

1. Pepsin produced no significant splitting of raw egg white under the experimental conditions employed. The hydrolysis effected by trypsin was slight; a preliminary incubation of the raw egg white with pepsin greatly facilitated the attack of the material by trypsin.

2. Preliminary periods of heating, up to 30 minutes in length, increased the degree of hydrolysis of egg white by trypsin. The extent of digestion of egg white by the successive action of pepsin and trypsin also varied directly with the length of the preliminary heating period to which the substrate was subjected.

3. These experimental results are interpreted as supporting the existence in raw egg white of an antitryptic agent which is slowly inactivated by heat.

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## ISOLATION OF MUCOITINSULFURIC ACID FROM CANINE GASTRIC JUICE\*

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Within the last few years considerable evidence has been accumulated in this laboratory justifying the assumption that a mucoprotein, which has been designated "dissolved mucin," is present among the organic constituents of pure canine gastric juice (Babkin, 1929, 1931; Webster, 1930, 1931; Webster and Komarov, 1932). The "dissolved mucin" was considered as distinct from the "visible mucus" which covers the surface of the gastric mucosa. It was demonstrated (Webster and Komarov, 1932) that different samples of filtered gastric juice obtained from different animals and under various conditions of stimulation yield, on treatment with acetone as a basis of procedure, a protein material identical in general properties and elementary composition (average percentage figures: C 53.75, H 6.97, N 13.84, S 1.29). Some of the sulfur (0.25 per cent) was present in the form of organically combined sulfates. The substances did not contain any phosphorus, did not reduce Fehling's or Benedict's solutions, but after boiling  $2\frac{1}{2}$  to 3 hours with 2 N  $\text{H}_2\text{SO}_4$  or N HCl, there was a reduction equivalent to 12.7 per cent glucose. All the general protein color reactions were positive. The consistency of the elementary composition of the different preparations was thought to prove that this substance is a body *sui generis* and a product of the normal secretory activity of the gastric glands. The low percentage of nitrogen, the presence of organically combined sulfates, the absence of phosphorus, and the considerable reduction after hydrolysis were considered to be sufficient evidence to classify this substance as a mucoprotein.

\* A preliminary communication was read at the meeting of the Royal Society of Canada, May 24, 1934.

According to the present day theory concerning the structure of mucoproteins, these substances should be regarded as complex proteins containing as a characteristic prosthetic group a conjugated sulfuric acid of the type of chondroitin- or mucoitinsulfuric acid (Levene, 1925). Therefore only isolation of this prosthetic group from the products of hydrolysis would be direct and indisputable evidence for classification of the protein in question as a mucoprotein. In the present investigation we believe that we have secured such evidence with regard to the mucoprotein ("dissolved mucin") of gastric juice. Three preparations having all the properties characteristic of Subgroup A of the mucoitinsulfuric acids, according to the classification of Levene, were isolated, two of them directly from the products of alkaline hydrolysis of freshly secreted and filtered gastric juice and one from the mucoprotein described by Webster and Komarov (1932).

#### EXPERIMENTAL

##### *Preparation of Mucoitinsulfuric Acid Directly from Gastric Juice*

Gastric juice was obtained by sham feeding from three healthy dogs with gastric fistula and esophagotomy. At 9 a.m. the stomach was usually empty but nevertheless it was washed out twice with warm water. The first 50 or 100 cc. of secretion obtained after sham feeding were discarded. Only water-clear, odorless secretion was used. It was collected every 15 minutes, immediately filtered through cotton wool, and placed in the ice box until all the daily secretion (about 500 to 800 cc.) had been collected. This required from 2 to 3 hours. The collected juice was filtered through Whatman filter paper No. 40, immediately neutralized with 5 N NaOH, concentrated under reduced pressure to one-twentieth of the original volume, and preserved for a few days at room temperature under toluene, until sufficient material was accumulated. Two samples of such material obtained from 1.5 and 2.5 liters of gastric juice were treated separately as follows: After removal of toluene by distillation the final concentrate was hydrolyzed for 3 days at room temperature with NaOH, which was added in the proportion of 2 per cent. The product of hydrolysis was then precipitated by 3 volumes of 96 per cent alcohol. Next day the precipitate was separated on the centrifuge, and

washed twice with 80 per cent alcohol, and twice with acetone. Acetone was removed *in vacuo* and the precipitate was dissolved in 20 cc. of water; the filtered solution was poured into 200 cc. of glacial acetic acid. A characteristic flocculent precipitate, which appeared instantaneously, was centrifuged, washed twice with 95 per cent acetic acid, and then with 96 per cent alcohol until practically all the acetic acid was removed. At this stage the precipitate, on being stirred in alcohol, tends to form a fine stable suspension. The addition of a few cc. of concentrated solution of NaCl in 80 per cent alcohol caused a good flocculation. The precipitate was then washed twice with 80 per cent alcohol containing traces of NaCl, then again with 96 per cent alcohol, with absolute alcohol, and with ether, and finally dried *in vacuo*. This product was not entirely soluble in water. It was extracted twice with 20 cc. of water; the combined extracts were concentrated to 20 cc. and precipitated again with 200 cc. of glacial acetic acid, the precipitate being carefully washed with alcohol and ether. All the residues, except the filtrates from glacial acetic acid precipitation, were combined, neutralized, concentrated, and then hydrolyzed again with 2 per cent NaOH for 2 days, the whole procedure being then repeated once more. A further, smaller yield of a product apparently identical with the first yield was obtained. Both were combined, dried in a vacuum desiccator over sulfuric acid in the presence of soda-lime, and then analyzed. From the first sample of 1.5 liters of gastric juice, 66.8 mg. of a substance (Preparation I), which on analysis was found to be a fairly pure sodium salt of mucoitinsulfuric acid, was obtained. This corresponds to a yield of 4.16 mg. per cent of free acid. From another sample of 2.5 liters of gastric juice 65 mg. of a substance (Preparation II) containing 5.6 per cent ash were obtained. Apparently it was a mixture of mucoitin and mucoitinsulfuric acid. The yield of free acid thus was about 2.5 mg. per cent. Difference in the yield and in the composition of the two preparations obviously is due to the fact that during such a long and complicated procedure some uncontrollable slight variations were unavoidable. Variations in the room temperature at the stage of alkaline hydrolysis and in the amount of NaCl added at the stage when washing with alcohol was carried out, should be particularly mentioned in this connection.

*Preparation of Mucoitinsulfuric Acid from Mucoprotein of  
Gastric Juice*

Mucoprotein used in this work was isolated from 15.4 liters of gastric juice exactly as in Preparation III, described by Webster and Komarov (1932). The substance had the same general properties and practically the same elementary composition: C 52.68, H 7.00, N 14.02, S (as organically combined sulfates) 0.37 per cent. 9 gm. of this substance, corresponding to 6.8 liters of gastric juice, were dissolved in 80 cc. of 2 per cent NaOH and treated further in the same way as the concentrates of gastric juice described in the previous section. In precipitating the product of alkaline hydrolysis with alcohol it was necessary to add 2 gm. of sodium chloride in order to cause a good precipitation. The final product was converted into a barium salt by dissolving in 20 cc. of water and adding saturated barium hydroxide solution up to the point at which the reaction just began to turn slightly alkaline to litmus. After centrifuging, the solution was filtered into an equal volume of absolute alcohol. The precipitate was washed on the centrifuge with alcohol in increasing concentrations and finally with absolute alcohol and anhydrous ether, and then dried in a vacuum desiccator over sulfuric acid in the presence of soda-lime. The total yield was 365 mg. When dry the substance was a heavy, snow-white powder. It is described below as Preparation III.

*Properties of Substances Isolated*

All three preparations showed identical general properties. As free acid and sodium salt (Preparations I and II) the substance is very easily soluble in distilled water, in aqueous alkalies, and in dilute mineral acids, giving slightly opalescent solutions. It is soluble in dilute acetic acid, but gives a very characteristic flocculent precipitate in 80 per cent or stronger acetic acid. It is insoluble in 80 per cent or stronger ethyl alcohol in the presence of small amounts of electrolytes; insoluble in acetone and ether. As barium salt (Preparation III) the substance is easily soluble in water and in weak solutions of hydrochloric acid, giving a perfectly clear solution; insoluble in 50 per cent or stronger ethyl alcohol; insoluble in acetone and ether. As free acid (Preparations I and II) or as Ba salt (Preparation III) the substance does not re-

duce Fehling's solution, but shows a strong reduction after preliminary hydrolysis with dilute mineral acids. With naphthoresorcinol and with  $\alpha$ -naphthol it gives positive tests for glucuronic acid (Tollens' and Goldschmiedt's tests). It does not give any of the general protein color reactions (negative biuret, xanthoproteic, Millon, Hopkins, lead acetate test for unoxidized sulfur) but gives a strongly positive Molisch test. It contains all the sulfur (nearly the theoretical amount) in the form of organically combined sulfates. Among the products of alkaline hydrolysis volatile acids were found to be present; the amounts, calculated on the assumption that the volatile acid is acetic acid, are very close to the theoretical. The substance did not contain any phosphorus detectable by the procedure of Fiske and Subbarow (1925) after moist digestion with  $\text{H}_2\text{SO}_4 + \text{HNO}_3$ .

#### *Composition of Substances Isolated*

*Preparation I*—15.4 mg. of substance gave on incineration 3.20 mg. of ash, which was entirely soluble in water, giving a neutral solution.  $\text{BaCl}_2$  caused a characteristic  $\text{BaSO}_4$  precipitate;  $\text{AgNO}_3$  in the presence of nitric acid gave only a very slight opalescence.

5.08 mg. of substance required 0.50 cc. of 0.02 N  $\text{H}_2\text{SO}_4$  for neutralization of  $\text{NH}_3$  (Kjeldahl). A duplicate determination gave identical results.

9.072 mg. of substance gave 3.1 mg. of  $\text{BaSO}_4$  after preliminary hydrolysis with N  $\text{HCl}$  in a sealed tube at  $100^\circ$  for 6 hours.

0.4064 mg. of substance reduced respectively 1.26, 1.26, and 1.28 cc. of 0.005 N  $\text{K}_3\text{Fe}(\text{CN})_6$  after preliminary hydrolysis with N  $\text{HCl}$  in a sealed tube at  $100^\circ$  for 6 hours, the Hagedorn-Jensen (1923) method being used.

Calculated. Ash 20.8, N 2.75, S 4.69; reducing power equivalent to 55.4% glucose

Theory for  $\text{C}_{23}\text{H}_{44}\text{O}_{29}\text{N}_2\text{S}_2\text{Na}_4$ . Ash 27.6, N 2.72, S 6.23

The composition of this substance evidently corresponds well with that of sodium salt of mucoitinsulfuric acid, according to Levene's theory of its structure.

*Preparation II*—20.6 mg. of substance gave on incineration 1.15 mg. of ash. The ash was readily soluble in water, giving a clear

neutral solution, which yielded a characteristic  $\text{BaSO}_4$  precipitate on addition of  $\text{BaCl}_2$  in the presence of dilute  $\text{HCl}$  and also a characteristic  $\text{AgCl}$  precipitate on addition of  $\text{AgNO}_3$  in the presence of dilute  $\text{HNO}_3$ .

5.328 mg. of substance required 0.61 cc. of 0.02 N  $\text{H}_2\text{SO}_4$  for neutralization of  $\text{NH}_3$  (micro-Kjeldahl). A duplicate determination gave identical results.

0.444 mg. of substance reduced respectively 1.38, 1.38, and 1.40 cc. of 0.005 N  $\text{K}_3\text{Fe}(\text{CN})_6$  after preliminary hydrolysis with N  $\text{HCl}$  in a sealed tube at  $100^\circ$  for 6 hours.

7.9 mg. of substance hydrolyzed as in the above analysis gave a heavy precipitate with  $\text{BaCl}_2$ ; quantitative analysis was lost.

Calculated. Ash 5.6, N 3.12, reduction equivalent to 56.7 glucose  
 " for ash-free substance. N 3.40, reduction equivalent to 60.0 glucose

Theory for  $\text{C}_{28}\text{H}_{48}\text{O}_{25}\text{N}_2\text{S}_2$ . N 2.98

" "  $\text{C}_{28}\text{H}_{48}\text{O}_{25}\text{N}_2$ . " 3.59

Thus this preparation is apparently a mixture of mucoitin and mucoitinsulfuric acid.

*Preparation III*—52.7 mg. of substance gave on incineration 17.6 mg. of ash, all of which was insoluble in dilute hydrochloric acid.

20.6 mg. of substance gave on incineration 6.9 mg. of ash, all of which was insoluble in dilute hydrochloric acid. Calculated, ash 33.4, 33.5; S 4.59, 4.61; base 19.65, 19.7 per cent.

For neutralization of  $\text{NH}_3$  (Kjeldahl)

4.9 mg. substance required 0.44 cc. 0.02 N  $\text{H}_2\text{SO}_4$

4.9 " " " 0.43 " 0.02 " "

5.675 " " " 0.49 " 0.02 " "

5.675 " " " 0.49 " 0.02 " "

Calculated. N 2.46

The carbon and hydrogen were determined by semimicrocombustion, this being kindly undertaken by Mr. S. C. Overbaugh, of the Laboratory of Organic Chemistry, McGill University. The filling of the combustion tube was as required for the combustion of substances containing N, S, and Cl. Reported, C 30.6, H 4.25, ash 33.2 per cent.

70.0 mg. of substance were dissolved in 25 cc. of water. The solution was perfectly clear.

10 cc., containing 28.0 mg. of substance, were heated with 2 cc. of 5 N HCl in a sealed tube for 10 hours at 100°. In about 1 hour the precipitate of BaSO<sub>4</sub> began to appear. 9.0 mg. of BaSO<sub>4</sub> were recovered without addition of BaCl<sub>2</sub> to the product of hydrolysis. Addition of BaCl<sub>2</sub> to the filtrate did not cause any precipitation. Calculated, S 4.41 (in the form of organically combined sulfates).

10 cc. of the solution, containing 28.0 mg. of substance, were heated for 10 hours at 100° in a sealed tube with 5 cc. of hot saturated Ba(OH)<sub>2</sub> solution. The contents of the tube were perfectly clear. Soon after the commencement of heating a yellow precipitate appeared. The product of hydrolysis was filtered directly into Pregl's (1930) micro-Kjeldahl apparatus, washed twice with CO<sub>2</sub>-free water, and 12 cc. of 50 per cent H<sub>2</sub>SO<sub>4</sub> then added. Distillation with steam was carried out for 30 minutes, the distillate being passed into an ice-cooled receiver, provided with a guard containing soda-lime. 4.25 cc. of 0.02 N NaOH were required to neutralize volatile acids. The blank was 1.55 cc. of 0.02 N NaOH. The distillate was carefully neutralized with sulfuric acid (slightly acid to litmus, but basic to Congo red) and treated with 2 cc. of 0.02 N AgSO<sub>4</sub> until there was no more precipitate; the solution was filtered when hot and the precipitate was washed three times with 5 cc. of hot CO<sub>2</sub>-free water. The total volume of the filtrate was about 40 cc. It was again distilled for 30 minutes with 10 cc. of 50 per cent sulfuric acid (freshly opened bottle of Merck's reagent). 2.84 cc. of 0.02 N NaOH were required to neutralize the volatile acids to phenolphthalein. The blank was 0.60 cc. of 0.02 N NaOH. Calculated, volatile acid (as acetic acid) 9.61 per cent.

0.98 mg. of substance reduced 2.50 cc. of 0.005 N K<sub>3</sub>Fe(CN)<sub>6</sub> (Hagedorn-Jensen) after preliminary hydrolysis with N HCl in a sealed tube at 100° for 6 hours. A duplicate determination gave identical results. Calculated, reducing power equivalent to 48.3 glucose.

Found. C 30.6, H 4.25, N 2.46, S 4.60, base 19.7, volatile acids equivalent to 9.61 acetic acid

Theory. C 27.8, H 3.48, N 2.32, S 5.30, base 22.7, acetic acid 9.91

Data obtained on analysis of our Preparation III show that this substance has a composition very close to that required by the theory for barium salt of mucoitinsulfuric acid, as established by Levene.



## DISCUSSION

The method used for isolation of mucoitinsulfuric acid from gastric juice was adopted as a result of another study which was carried out on commercial gastric mucin (also with a view to isolation of mucoitinsulfuric acid), and which will form the subject of a special communication. By the use of different methods it was possible to obtain from commercial gastric mucin preparations of mucoitinsulfuric acid having all the characteristics of that particular group of mucoitinsulfuric acids which was classified by Levene as Subgroup A. The elementary composition and general properties were practically identical, but the yield varied from 4.2 to 26.0 per cent, depending on the method used. Since the source of commercial gastric mucin as certified by the manufacturers was hog gastric mucosa and the gastric mucin as manufactured is only a by-product in the preparation of commercial pepsin, it was thought that possibly the dissolved mucin of gastric juice is also a derivative of this group of mucoitinsulfuric acids. The distinctive peculiarity of this group of conjugated sulfuric acids, as emphasized by Levene, lies in the solubilities of free acids and their salts. As free acids and as barium salts they are very soluble in water and are precipitated from aqueous solutions only by a large excess of glacial acetic acid, and when thus precipitated the substances come down in the form of very light floccules, in contradistinction to the substances of the second subgroup. These, under the same conditions, form a heavy gelatinous mass, and only a slight excess of acetic acid is required to produce such a precipitation. Another important distinction of the substances belonging to the second subgroup is that their barium salts are practically insoluble in water. The method outlined in the experimental part of this study is based entirely on these characteristic features.

Our preparations obtained from gastric juice have identical general properties. Unfortunately only one of the preparations was obtained in sufficient quantity for a more complete analysis. However, quantitative estimation of nitrogen and of reducing power after preliminary hydrolysis could be carried out in all cases. These data, when supplemented by a negative biuret test and a negative test for phosphorus as well as by a positive naph-

thoresorcinol test and a positive test for organically combined sulfates, are entirely sufficient for identification. We wish to emphasize here the importance of quantitative determination of reducing power after preliminary hydrolysis for identification of mucoitinsulfuric acid. In our experience seven different preparations of mucoitinsulfuric acid, identification of which was supported by a complete elementary analysis, had reducing power equivalent on the average to 60.0 per cent glucose content, with observed variations of from 58.0 to 61.6 per cent; hydrolysis was carried out in sealed tubes with  $N$  HCl at  $100^{\circ}$  for 6 hours, and

TABLE I

*Analytical Data for Preparations of Mucoitinsulfuric Acid from Gastric Juice*

Nitrogen and sulfur are expressed in per cent; reducing power in per cent of glucose content; yield in mg. per cent of gastric juice used for isolation. Preparations I, II, and III were analyzed as Na salt, free acid, and Ba salt respectively. All the values have been calculated for the free acid.

Preparation No.....	I	II	III	Theory
N.....	2.95	3.40	3.06	2.98
S.....	5.04		5.73 5.40	6.81
Reducing power.....	59.4	60.0	60.0	
Yield.....	4.1	2.5	4.32	

determination of sugar by the method of Hagedorn-Jensen, preliminary precipitation with zinc sulfate being omitted.

In spite of the fact that our preparations were analyzed as different compounds of mucoitinsulfuric acid—sodium salts as free acid and as barium salt—the results of the analysis show that in all these cases we are dealing with the same substance. Table I represents analytical data calculated for free mucoitinsulfuric acid. The figures for nitrogen, sulfur, and reducing power are identical or within the limits of experimental error.

The striking feature of the more completely studied Preparation III, isolated from the mucoprotein of gastric juice, is its surprisingly high degree of purity, which approximates to the best known preparations of chondroitinsulfuric acid. Sulfur was determined

three times; twice in the ash and once after hydrolysis in a sealed tube with  $\text{N HCl}$ . The first two determinations gave identical results: S 4.60 per cent; the third gave S 4.41 per cent. Volatile acids after hydrolysis with  $\text{Ba(OH)}_2$  also gave almost the theoretical value for acetic acid. Unfortunately the nature of the amino sugar could not be exactly determined, owing to the lack of material. However, a few mg. of crystalline material were isolated as hydrochloride from the products of acid hydrolysis in the samples used for the determination of organically combined sulfates. This substance reduced Fehling's solution, did not melt at  $200^\circ$ , and turned black at  $220^\circ$ , but it was contaminated with some inorganic material. These data might be considered as suggestive that the sugar is probably chitosamine. Another consideration in favor of such a conclusion is that so far chondrosamine has been isolated only from chondromucoids, which occur in the connective tissue of various organs (aorta, tendons, cartilage, sclera) and have never been found in animal fluids, mucous membranes, and their secretions or in any other tissues, except connective tissue (Levene, 1925). Therefore we believe that the substance described in this communication must be termed mucoitinsulfuric acid in accordance with the classification of Levene, although the direct proof—identification of amino sugar—is at present not available. The importance of identification of amino sugar in this preparation cannot be overestimated. Its indisputable identification as chitosamine would considerably substantiate Levene's theory of the structure of mucoitinsulfuric acid and of mucoproteins in general. However, even as the question now stands, our data cannot be reconciled with Schmiedeberg's (1920) view that only chondromucoids contain a carbohydrate complex of the structure of a sulfuric ester of a complex carbohydrate, and that true mucins do not contain sulfuric acid in their carbohydrate compounds. On the other hand, our data are entirely concordant with the postulates of Levene's theory.

However important the exact nature of the substance described in this paper may be in relation to the theory of mucoproteins, for the main purpose of this investigation it is immaterial, since in any case—whether it is chondroitinsulfuric acid or mucoitinsulfuric acid—the protein containing this carbohydrate complex in a quantity allowing of its isolation in an analytically pure state in

the proportion of 4 per cent of the original material must unquestionably be regarded as a mucoprotein. In this connection it is very interesting to compare the yield of the substance, when isolated directly from the gastric juice and from the mucoprotein obtained from the juice. The yield in the latter case was 4.32 mg. per cent, as compared with 4.1 mg. and 2.5 mg. per cent obtained directly from the gastric juice. This indicates clearly that mucoitinsulfuric acid is present in the gastric juice only as an integral part of mucoprotein—in the form of “dissolved mucin”—and not as free mucoitinsulfuric acid which might be liberated from the surface mucus as the result of peptic digestion. Peptic digestion of the surface mucus, however, is very improbable for the reason that the gastric juice used in the investigation was filtered within 15 to 30 minutes after being secreted.

#### SUMMARY

1. Two preparations, identified as sodium salt of mucoitinsulfuric acid and free mucoitinsulfuric acid respectively, were isolated from the products of alkaline hydrolysis of two samples of freshly collected and immediately filtered canine gastric juice.

2. Barium salt of mucoitinsulfuric acid was isolated from the mucoprotein, “dissolved mucin,” of gastric juice.

The writer wishes to express his thanks to Dr. B. P. Babkin who directed this work.

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## STUDIES ON THE ADRENAL

### VIII. A SIMPLE PREPARATION OF THE ADRENAL CORTICAL HORMONE SUITABLE FOR ORAL ADMINISTRATION

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Of all the available endocrine products, none presents such difficulty and expense in its preparation as the adrenal cortical hormone. The availability of a relatively simple method for the preparation of this hormone would make possible its therapeutic application in adequate dosage and permit its use in the elucidation of the many biological problems in which the adrenals are involved. In the present paper we shall describe a simple method for obtaining the hormone in a form suitable for oral administration, which, when administered in relatively small amounts, serves as a complete replacement therapy in bilaterally adrenalectomized animals (rats and dogs).

In seeking a method for concentrating the hormone, we attempted to adsorb the hormone selectively on various agents, utilizing the methods which have been so fruitful in the purification of the enzymes and vitamins. It was soon found that a number of agents—charcoal, fullers' earth, metallic oxides, and permutit—readily adsorbed the hormone. This adsorption is particularly complete in the case of activated charcoal, when the hormone is present in neutral aqueous solution. The liberation of the hormone from this combination in the gastrointestinal tract permits

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its absorption and utilization by the body. Animal experiments demonstrated many advantages of this oral method of therapy over the parenteral administration which heretofore has been the method of choice.

*Source of Glandular Materials*—In a previous paper (4) we described the preparation of an extract suitable for parenteral injection. Except for the use of pig glands (instead of beef glands) and a slightly modified mode of extraction with acetone (to be described subsequently) our original method has proved entirely satisfactory and superior to those described by previous workers.<sup>1</sup> The comparison of the yield of hormone from glands derived from various commercially available animal species (sheep, cattle, pig, and calf) led us to adopt pig glands as most suitable. Although much smaller than beef glands, which have been most widely used in the past, the yield of hormone per unit weight of pig glands is greater than that from cattle. This may in part be due to the relatively smaller size of the medulla compared to the whole gland in the pig as compared to that in cattle. Glands derived from sheep or calves are also probably rich in the hormone, but the difficulty of properly collecting and preserving them renders their use unsatisfactory.

As previously shown (4), preservation of the glands by freezing is necessary for preventing destruction of the hormone and the formation of lipoidal and other autolytic products which contaminate the final extract. We have repeatedly confirmed this on glands obtained under various conditions. To illustrate the course of this destruction various portions of a batch of glands obtained from one source were extracted and assayed. One portion of the glands was removed from the carcasses of freshly slaughtered animals and dropped into liquid air. These glands were immediately extracted with acetone as previously described (4). The extract thus obtained was found to contain 500 rat units (as defined in previous papers (4, 5)) per kilo of the original whole glands. A second portion of the glands was frozen with dry ice at the abattoir and packed in this dry ice for 2 days before extraction. The extract obtained from these glands contained 100 rat units per kilo

<sup>1</sup> The adsorption method described in the present paper permits the recovery of any hormone lost in applying the procedures previously described (4) for preparing the extracts.

of whole glands. A third portion of the glands was placed in the refrigerator of the abattoir and sent frozen to the laboratory on the following day. The extract prepared from these glands contained only 10 rat units per kilo of glandular material. A fourth portion of the glands was sent unfrozen to the laboratory. The extract prepared from this material was found to contain only a trace of activity.

The deleterious effects of poor preservation of adrenal glands on the hormone yield, which have been verified by us many times, are obvious from the experiment described above. For practical purposes the first method indicated above for obtaining glands is impractical where large quantities of glands are utilized, and hence we have in general adopted the second method cited. The glands are removed at the abattoir, well defatted, and instantly frozen by the so called "monel-metal quick freeze" process. The glands are then packed in dry ice (carbon dioxide snow) and shipped to the laboratory where they arrive 2 days later. This method of procuring the glands proved much more satisfactory than obtaining them locally where the quick freezing process was not available.

Where only a small amount of glandular material is available, it is advisable to transfer the glands directly from the animal body into acetone and immediately macerate them. By this method we have been able to demonstrate the presence of the hormone in the interrenal body of fishes (6). In the case of rat glands removed at operation and extracted in this way about 2000 rat units of hormone per kilo of glands were obtained. The mode of collection of the glands is of fundamental importance in determining the ultimate potency of the hormone. The presence of a large lipoidal precipitate in the last stage of the process for preparing extracts (4) indicates poor preservation of the glands. This lipid also adsorbs appreciable amounts of the hormone which can, however, be recovered by alternately dissolving the lipid in 0.1 N alkali and precipitating it by the addition of concentrated hydrochloric acid. The hormone remains in the aqueous solution.

*Extraction of Glands*—The adrenal cortical hormone, although not soluble in solvents such as benzene or ether when purified, is firmly attached to the lipids of the adrenal cells and is removed only when these lipids are simultaneously removed. Thus attempts to extract the hormone from the glands by the use of dilute acid



or alkali have been unsuccessful in our experience. Dehydrating the fresh, ground glands *in vacuo* at 37° destroys the cellular structure and permits extraction of the hormone by water. Unfortunately, this method of extraction was not practical due to the technical difficulties of further purifying the aqueous extract thus obtained.<sup>2</sup> It is, therefore, necessary to use a lipid solvent (acetone, alcohol, or ether) which extracts the adrenal lipids and incidentally the cortical hormone. We have found (4) acetone to be by far the most practical solvent, for it not only extracts the hormone efficiently but removes less extraneous matter, and hence leads to the easier production of a relatively pure product.

On receipt of the frozen glands they are finely ground into 2.5 to 3 times their volume of technical acetone. The glandular material is then thoroughly macerated and shaken at intervals for at least several hours. This operation is carried out at room temperature for the acetone apparently inhibits the enzymic activity which, as we have seen, acts so deleteriously in the intact unfrozen glands.

The supernatant solvent is now poured off from the glandular material. This glandular residue is refluxed three times with approximately one-third of the acetone which was poured off, the mixture being pressed after each refluxing. In this way one carries out the three refluxings with the 2.5 to 3 volumes of acetone originally used and avoids the use of the excessive volume of solvent which we previously suggested (4).

By the method described above about 95 per cent of the hormone present in the glands is extracted. A fourth and fifth reextraction and refluxing gives only small amounts of the hormone. Since our most purified preparations of the hormone are soluble in acetone, it would be improbable that this solvent would fail, in subsequent extractions, to dissolve the hormone. Moreover, attempts to extract any residual hormone with ethyl alcohol, ether, ethylene dichloride, acid, and alkali all failed to yield any appreciable amounts of the hormone.

*Preparation of Charcoal-Hormone Combination*—Since the adrenal cortical hormone is not completely adsorbed from aqueous

<sup>2</sup> We are indebted to Dr. Fitzgerald Dunning and Dr. Wilton C. Harden of Hynson, Westcott and Dunning for kindly dehydrating the glands and making the aqueous extracts.

acetone solution by charcoal, the acetone must be removed before proceeding to adsorb the hormone on charcoal. After being chilled in the ice chest, the acetone extract, obtained as described in the preceding section, is filtered to free it from the separated lipids. The clear filtrate is distilled *in vacuo* at 35–40° until all the acetone is removed. The completeness of this removal may be determined from the specific gravity of the residual aqueous fluid, which should be over 0.99 at room temperature. After being chilled in the ice chest again, the solution is again filtered to remove the separated lipids.<sup>3</sup> The filtrate is carefully neutralized with an aqueous solution of NaOH until neutral to litmus paper (pH 7.0) and shaken at intervals with activated charcoal (norit, or decolorizing carbon). 1 gm. of charcoal is used for every 200 gm. of glandular material originally extracted. After some hours (6 or more, depending on the efficiency of the shaking) the carbon is collected on a Buchner funnel. The filtrate contains only a trace of hormone, but this can be recovered by treatment again with charcoal.

The charcoal-hormone combination thus prepared proved satisfactory when used on rodents, but proved irritant to the stomach when tested on adrenalectomized dogs. Although protecting against the development of symptoms of adrenal insufficiency, the presence of epinephrine and possibly other impurities adsorbed on the charcoal ultimately caused retching and vomiting, which prevented its further administration. To remove these impurities the charcoal-hormone combination is suspended in about 3 times its volume of 10 per cent hydrochloric acid (1 part of 33 per cent HCl to 10 parts of water). After thorough agitation, the suspension is filtered on a Buchner funnel. The charcoal is then suspended in about 3 times its volume of 0.1 N sodium hydroxide solution, agitated, and again filtered. It is then again washed

<sup>3</sup> The adrenal cortical hormone attaches itself readily to lipids and is carried down to some extent whenever lipids are precipitated. The small amount of hormone thus precipitated may be recovered by dissolving the lipid in alcohol or acetone, diluting with water to precipitate the lipid, distilling off the alcohol or acetone, and adsorbing the hormone on charcoal as described in the text. For experiments on rodents we have found it preferable to dissolve the lipid in ether, evaporate to dryness, extract with ethyl alcohol, pour the alcoholic solution over the food, and dry until the alcohol has evaporated. Food thus prepared will maintain normal growth in adrenalectomized rats.

with 3 volumes of 2 per cent hydrochloric acid and finally washed with a little distilled water and filtered. It is now ready for use. The charcoal-hormone combination may also be effectively freed of adsorbed impurities by washing on the Buchner funnel with dilute ammonia, water, and ethyl alcohol, respectively. These reagents elute an inappreciable amount of the hormone.

The amounts of acid and alkali used for washing the charcoal are relatively small, and hence do not remove an appreciable quantity of the hormone. The trace removed can be reclaimed by neutralizing the washings and reabsorbing on a little charcoal. The charcoal-hormone compound, when administered therapeutically, comes into contact with many times its own volume of gastrointestinal juices which succeed in eluting the hormone from its adsorbant. Since the charcoal remains in contact with the alkali for only a few minutes during the washing, no appreciable amount of the hormone is destroyed by this procedure.

For administration to rodents, dogs, or other experimental animals, the desired amount of charcoal is thoroughly admixed with the food. For clinical application it may be compressed into tablets coated with sugar or other protective covering to avoid access of air and possible oxidation of the hormone.

*Biological Tests*—The therapeutic efficacy of the adrenal cortical hormone, when adsorbed on charcoal, was demonstrated by feeding it to adrenalectomized rats and dogs. Rats, adrenalectomized under phanodorn anesthesia<sup>4</sup> as previously described (2), were allowed access to their regular diet (a powder containing cornmeal, whole milk powder, alfalfa meal, casein, sodium chloride, and calcium carbonate) into which was thoroughly incorporated 0.1 gm. of the charcoal-hormone combination per rat per day. The control animals were maintained under similar conditions but fed on a mixture containing charcoal on which no hormone had been adsorbed. These control animals died from adrenal insufficiency within a week following operation. The treated animals grew

<sup>4</sup> We wish to thank the Winthrop Chemical Company, Inc., the Abbott Laboratories, and Eli Lilly and Company for generous supplies of phanodorn, nembutal, and amytal, respectively, the anesthetics used in this study. We are indebted to Merck and Company, Inc., for supplying us with a generous amount of the charcoal-hormone preparation made according to our directions.

normally and survived as long as an adequate amount of the hormone was added to their food. When the charcoal-hormone combination was omitted from the food, the animals followed the usual course of adrenal insufficiency and died (2). Ten different batches of the charcoal-hormone combination have been assayed on groups of six rats each with identical results. A typical assay is reproduced in Table I.

TABLE I

*Typical Assay Results on Group of Twelve Rats Adrenalectomized at Age of 30 Days*

To Rats 1 to 6 was administered a total daily dose of 0.6 gm. (dry weight) of the charcoal-hormone combination corresponding to 120 gm. of whole pig adrenal glands. The treatment was discontinued on the 7th day after operation. Rats 7 to 12 served as untreated controls.

The values listed are body weights measured in gm.

	Rat No.	Days after adrenalectomy											
		1	2	3	4	5	6	7	8	9	10	11	12
Treated	1	34	38	41	43	45	46	49	52	53	54	54	D*
	2	36	37	40	45	48	50	53	55	55	54	D	
	3	36	39	43	46	48	50	52	54	53	52	D	D
	4	34	36	37	40	43	46	48	51	52	50	50	
	5	40	39	44	47	49	54	55	57	58	56	D	D
	6	38	40	41	44	47	50	52	56	58	57	57	
Controls	7	38	37	39	39	38	D						D
	8	36	33	32	D								
	9	37	38	39	38	38	39	D					
	10	36	34	31	D								
	11	43	42	42	41	42	41	D					
	12	35	35	36	37	37	D						

\* D indicates death of animal.

In order to demonstrate the adequacy of treatment over long periods of time by the hormone adsorbed on charcoal, a group of four adrenalectomized rats was given 0.4 gm. of the charcoal-hormone combination, daily, mixed with the food. These animals grew normally to their adult size and manifested, as far as could be observed, all the signs of perfect health. In Fig. 1 are reproduced the growth curves of these rats treated for 60 days following adrenalectomy. Under adequate treatment the rats never devel-

oped any failure to respond to the hormone, such as has been claimed to occur, and which has been attributed to the develop-

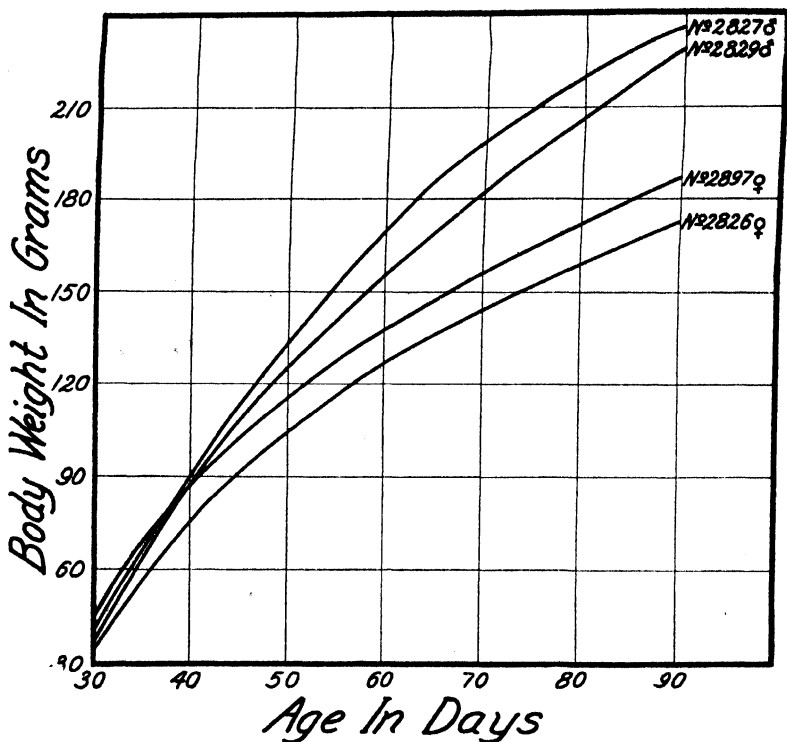


FIG. 1. The growth curves of four rats, adrenalectomized at 30 days of age under phanodorn anesthesia. The four animals received daily 0.4 gm. of the charcoal-hormone combination (equivalent to 80 gm. of whole pig adrenal glands) which was mixed with the ration described in the text. Lettuce was added twice weekly. At the age of 90 days, when the animals had reached adult size, treatment was ceased and the animals gradually developed the symptoms of adrenal insufficiency and died within the course of the 3 weeks following cessation of treatment. At autopsy no regenerated adrenal cortical tissue or accessory glands were demonstrable. The lungs were normal and there were no evidences of any disease other than the typical findings of adrenal insufficiency.

ment of an antihormone (1). In our own experience, the apparent development of a failure to respond to the hormone is due to treat-

ment with an inadequate dose of the hormone. This results in a chronic insufficiency, the nature of which shall be described elsewhere.

The potency of the charcoal-hormone combination was also tested on adrenalectomized dogs. In these experiments the following technique was employed. Normal mongrel adults were doubly adrenalectomized under spinal anesthesia, as previously described (2). They were given an amount of the charcoal-hormone combination three times daily for varying periods (as indicated in the fourth column of Table II) which sufficed to main-

TABLE II

*Effect of Oral Administration of Charcoal-Hormone Combination on Survival of Adrenalectomized Dogs*

Each gm. of the charcoal preparation corresponded to 200 gm. of whole pig adrenal glands.

Dog No.	Sex	Post-operative weight	Average daily dose (dry weight) of charcoal-hormone combination administered	Period of treatment following operation	Postoperative survival period
		kg.	gm.	days	days
1	♂	7.05	2.5	10	15
2	♂	8.00	3.0	8	10 (Killed)
3	♀	11.75	2.0	28	28 (Accidentally killed)
4	♂	8.1	3.0	30	44
5	♂	12.45	3.0	30	36

tain them in normal health. The charcoal-hormone combination was mixed with a bolus of food and fed in three equally divided doses three times daily. At no time were any injections of extract, saline, or other adjuvant medication utilized. At the end of the period of treatment the animals had maintained or gained over their preoperative weights and appeared active and normal in every respect except Dog 2. Dog 2 was killed on the 10th day following operation because of the development of a trophic sore resulting from a paralysis following spinal anesthesia. Dog 3 was accidentally killed on the 28th day following adrenalectomy. After the cessation of the treatment the other animals developed the

usual signs of insufficiency and died on the dates indicated in the last column of Table II. Careful microscopic examination of the tissues at the adrenal sites revealed no remnants of adrenal tissue.

In Table III are reproduced the survival periods of thirty dogs adrenalectomized under the identical conditions and by the same operator as the dogs of Table II and given no postoperative treatment. These operations were carried out during the same 6 month period during which the animals of Table I were adrenalectomized, and should serve as adequate controls for the experiments of Table I. Comparison of the results of Tables II and III demonstrates the ability of the charcoal-hormone combination to maintain life in adrenalectomized dogs.

No attempt has been made to determine the degree of insufficiency from which dogs may be revived by oral treatment with the

TABLE III  
*Survival Period of Control Dogs after Bilateral Adrenalectomy under Spinal Anesthesia*

Days following operation.....	1	2	3	4	5	6	7	8
No. of animals dying on day indicated.....	4	6	5	4	3	5	2	1

charcoal-hormone combination, for the nausea and vomiting make oral therapy impossible in severe adrenal insufficiency. However, in several cases in which inadequate doses of hormone had resulted in the early symptoms of insufficiency (weakness, loss of weight, drop in body temperature, and refusal to eat) the introduction into the stomach of a suspension of the charcoal-hormone combination in water resulted in the disappearance of these symptoms and recovery of the dog. Oral therapy by the charcoal-hormone combination would thus appear to be satisfactory except when the vomiting of adrenal insufficiency makes it impossible for the animal to retain the charcoal.

#### DISCUSSION

In a previous study of the efficacy of oral as compared to parenteral administration of the cortical hormone (5) it was found that when extract was administered in a single daily dose about 5 times

as much hormone was necessary for oral as for parenteral therapy. We were astonished to find, therefore, in the case of rats, that approximately the same equivalent of adrenal glands administered in the form of the charcoal combination as was required for intraperitoneal injection sufficed to keep these animals in perfect health.

This discrepancy in the different dosages required when the hormone is administered orally in the form of extract and in the form of the charcoal-hormone combination is explained as follows: When the cortical extract is administered intraperitoneally in a single daily dose, part of it is probably immediately destroyed in the body or excreted in the urine (3). The same amount of hormone given in divided doses leads to much more efficient utilization by the body. Hence, a smaller amount of hormone is necessary to keep adrenalectomized animals alive when administered in divided doses than in a single dose. In the treatment of rats with the charcoal-hormone combination, as described above, the hormone was being administered almost continuously in infinitely small doses, and any destruction by the gastrointestinal tract was compensated for by the more efficient mode of utilization. In the case of dogs, their habits of periodic feeding render the utilization of the hormone less efficient than in the case of the rat.

The charcoal-hormone combination is by far the simplest preparation of the adrenal cortical hormone hitherto described capable of maintaining life in adrenalectomized animals. The advantages of oral therapy are manifold. The presence of impurities in extracts renders their parenteral administration dangerous. For experimental studies, these impurities often lead to results which are attributed to the hormone (7).

The charcoal-hormone preparation may also be used for preparing extracts suitable for parenteral administration. Elution of the hormone by aqueous pyridine, phenol, etc., serves as a more efficient method for preparing extracts than has been heretofore available.

Clinically the oral mode of therapy as advocated in this paper should offer many obvious advantages and replace the use of extracts except in severe crises. Doses as small as 0.2 gm. of the charcoal-hormone combination conveniently administered in pill or tablet form would correspond to 40 gm. of the whole gland.



The frequent administration of small doses orally should give more effective results than the necessarily infrequent injections previously employed.

The idea of administering a hormone in the form of an adsorption compound, as advocated in the present paper, is generally applicable in all cases where the free hormone is effective orally and is eluted from its adsorption compound by the gastrointestinal juices. An analogous method for eliciting the estrogenic effect of urine has been found successful and will be described elsewhere.

#### SUMMARY

A simple method is described for adsorbing the adrenal cortical hormone on charcoal. The product thus obtained has been used successfully for maintaining life in adrenalectomized animals. The many advantages of this form of oral therapy over the parenteral administration of extracts are indicated.

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## A NOTE ON THE PREPARATION OF STARCH SUBSTRATES FOR AMYLASE DETERMINATIONS

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In a recent communication (1) a sensitive precision method of estimation of amylolytic activity applicable to human serum has been reported. In order to avoid delay in preliminary preparation of the starch substrate, particularly where a series of observations in close succession is to be made, a modification was developed which consisted essentially of storage at 25° (after autoclaving as usual) of the solution of 3 gm. of soluble starch in 15 ml. of  $\text{M}$  sodium acetate and 45 ml. of water in the muslin-capped flask regularly used, followed by heating just to the boiling point before proceeding with the preparation as usual. Evaporation up to this point is unimportant quantitatively, as dilution to a final volume of 100 ml. follows (with certain other inclusions as described previously (1)). It is by no means obvious, however, that an equivalent substrate is obtained in this way; which is definitely not the case if instead the solution were stored in a refrigerator at 0–5°.

In the previous work (1) it was found convenient to take  $1/\omega$  as the index of activity of digestion mixtures, where  $\omega$  is the time (in hours since mixing) such that a 7.5 per cent change in viscosity has occurred in the last three-fourths of this time. Relative values of  $\omega$  with similar mixtures, with the same enzyme solution (pancreatin), were obtained by division of  $\omega$  found for a given mixture by the corresponding value for a mixture with *regularly prepared* substrate. Thus preparations from starch solutions stored 2 days at 25° were compared with the standard preparation, two in each of two experiments, giving respective values of *relative*  $\omega$ , 1.002, 1.032, 0.963, and 1.008 (mean = 1.001, and average deviation = 0.019, approximately). Similarly, with storage for 5 to 6 days, the mean of four such evaluations was 1.010 with a.d.  $\cong$  0.030.

In contrast with this, two preparations with starch solution stored 2 days in a refrigerator (0–5°) gave relative  $\omega$  values of 0.888 and 0.873, respectively.

In conjunction with a simple method of estimating  $\omega$ , which has been described (1) (a modified pantograph being used), there is automatically indicated a quantity,  $y_1$ , defined as the relative viscosity of the mixture as estimated from the digestion curve at the time,  $\omega/4$ . In the first experiments described above the mean and a.d. of  $y_1$  for the standard preparation were 2.499 and 0.009, while for 2 days storage at 25°,  $\bar{y}_1 = 2.548$  with a.d. = 0.009, and for 5 to 6 days storage,  $\bar{y}_1 = 2.542$  with a.d. = 0.021. In contrast again the preparations after 2 days storage in the refrigerator gave  $\bar{y}_1 = 2.646$  and 2.712, respectively. All digestions were followed in triplicate.

It appears, accordingly, that storage, for 5 days or less at 25°, of the preparation after autoclaving, followed by heating just to the boiling point before continuing as in the standard technique, is an admissible substitute. However, the proper value of  $y_1$  should be used if correction for viscosity elevation is required, as described (1) for the case where human serum<sup>1</sup> is used in the digestion mixture.

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<sup>1</sup> A unit of pancreatic amylase concentration based on a reproducible standard has been described (2) previously, against which the present system is standardized. It is worthy of note that this seems to be very nearly 10 times that used by Elman and his coworkers (3). Thirty-six combined normal values given by them for human serum (3) have a mean of 5.43 and relative standard deviation of 13.4 per cent, in comparison with which our first nine observations (1) gave a mean of 0.534 with relative standard deviation of 14.4 per cent.

# PIGMENTS OF THE MENDELIAN COLOR TYPES IN MAIZE. CHRYSANTHEMIN FROM PURPLE- HUSKED MAIZE\*

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One of our former papers (1) reported that isoquercitrin, a yellow flavonol glucoside, was obtained from brown-husked maize, one of a series of color types whose heritable behavior has been determined by Emerson at Cornell University. It was pointed out that the flavonol and anthocyanin groups of plant pigments are involved in the genetic relations of the six main color types and that in all probability the pigment of the purple-husked type would prove to be a cyanidin monoglucoside or the corresponding anthocyanin of isoquercitrin.

In obtaining chrysantheamin chloride from purple-husked maize, the isolation and identification of which is reported in this paper, we have substantiated a prediction with regard to the identity of this coloring matter and in addition we have brought up again the question of the origin of anthocyanins in plants. According to the researches of Everest (2) and of Willstätter and Mallison (3), the anthocyanidins are reduction products of flavonols. Thus, from the three more widely occurring flavonols, kempferol,  $C_{15}H_{10}O_6$ , quercetin,  $C_{15}H_{10}O_7$ , and myricetin,  $C_{15}H_{10}O_8$ , we would expect to obtain the corresponding anthocyanidins, pelargonidin,  $C_{15}H_{10}O_5$ , cyanidin,  $C_{15}H_{10}O_6$ , and delphinidin,  $C_{15}H_{10}O_7$ . *In vitro*, cyanidin has been obtained from quercetin by reduction (3), but there is as yet no conclusive evidence showing whether the anthocyanins (anthocyanidin glucosides) are derived in the plant directly from preexisting homologous flavonol glucosides or independently (4, 5).

\* Contribution No. 246 from the Food Research Division.

In a number of cases coexisting flavone and corresponding anthocyanin pigments have been isolated and identified. Everest (2) has shown the coexistence in purple-black pansies of the closely related pair myricetin and delphinidin. From the red rose (6, 7) and the wallflower (8, 9), quercetin and cyanidin have been obtained. The last two pigments have also been isolated from Jonathan apples by the senior author.<sup>1</sup>

Although the cases cited support the theory that anthocyanins are formed *in vivo* from corresponding flavonol glucosides by reduction, other instances yield contradictory evidence. Thus, the flavonol, kempferol, and the anthocyanidin, delphinidin, instead of the expected pelargonidin have been isolated from *Delphinium consolida* (10, 11). Another instance is the snapdragon, *Antirrhinum majus*, from the flowers of which luteolin and apigenin (12) and the unrelated cyanidin glucoside, antirrhinin (9), have been isolated. This is the only case excepting maize in which the pigments occurring in plant material of known genetic constitution have been investigated chemically.

Summarizing the available evidence for and against the reduction theory, we find five instances in which flavonols and their corresponding reduction products or anthocyanidins have been found in the same plant and two instances in which unrelated anthocyanidins have been identified along with the flavones (here used in the restricted sense to indicate lack of hydroxyl in position (3)) or flavonols. One of the latter cases, namely that of *Delphinium consolida*, has been discussed in a previous paper (1) and this discussion indicates the complexity to be expected and the possibility that more than one flavonol may be involved.

In some cases where a flavonol is reduced to its corresponding anthocyanidin it is not improbable that all or most of the preexisting flavonol is so converted, leaving behind any other flavones that happen to be present, such as luteolin and apigenin, which in most instances are converted to anthocyanidins less readily owing to the lack of hydroxyl in position (3). At any rate, we cannot assume that because a certain flavonol is not found coexisting with its homologous anthocyanidin, the latter was formed independently rather than by way of the flavonol. As mentioned, the particular

<sup>1</sup> Unpublished.

flavonol in question may have been fully converted into anthocyanidin or else it may have escaped attempts at isolation, especially if other flavones or flavonols were present to increase the difficulty of separation.

The results obtained from a study of the pigments in the brown and purple types of maize favor the possibility of an interrelationship of the flavonols and anthocyanidins which is based on the formation of one from the other by reduction of their corresponding glucosides. Heretofore only the free flavonols have been studied in this connection, whereas in the case of maize there is agreement not only between the flavonol and anthocyanidin, which are aglycones, or non-sugar portions of the molecule, but also agreement as to the sugar residue involved and its probable point of attachment. Both compounds are similarly constituted in that they yield on hydrolysis 1 molecule each of the aglycone and glucose. Robinson (13), who synthesized chrysanthemin chloride, has left no doubt that the sugar residue is attached to position (3). While Perkin (14) concluded the sugar in isoquercitrin is attached at any one of three points in the flavonol structure, he also pointed out that isoquercitrin is constituted like quercitrin, which has its sugar residue attached to position (3), but on hydrolysis yields rhamnose instead of glucose. It seems reasonably certain, therefore, that glucose is probably located at the same position in isoquercitrin as in chrysanthemin.

#### EXPERIMENTAL

The material used in this investigation represents one of the general color types recognized as purple in the Cornell experiments. In the series of six main color types the intensity of color runs as follows: Type I purple, Type II sun-red, Type III dilute purple, Type IV dilute sun-red, Type V brown, Type VI green. Full details of the genetic relations of these types have been published by Emerson (15), to whose memoir the reader is referred. Here it may suffice to say that purple is a color type possessing the genetic formula AA BB P1P1, and differing from other types in developing some purple color even in seedlings grown in the dark. At maturity nearly all parts are more or less purple including the culm, brace roots, leaf sheaths, husks, and staminate inflorescence. Material of this type was furnished by Professor Emerson.

*Preparation of Chrysanthemin*

*Extraction*—Ground, air-dried husks weighing 7800 gm. were extracted in a large percolator first with U.S.P. ether to remove fats and waxes, then with 0.5 per cent methyl alcoholic hydrogen chloride. The extract (25 liters) was evaporated under reduced pressure to approximately 3.5 liters and placed in an ice box for several days to permit the separation of less soluble impurities. After filtration, the clear, deep red solution was treated with 5 volumes of ether, causing the separation of the bulk of pigment as a dark gummy residue. After this was washed with ether, the precipitate was redissolved in methyl alcohol, the solution filtered from insoluble material, concentrated *in vacuo*, and treated again with ether. The gummy precipitate was dissolved in cold 0.01 per cent aqueous hydrochloric acid and the mixture filtered through talc to remove resinous impurities.

Addition of neutral lead acetate solution precipitated the anthocyanin as a bluish violet salt. This was collected, washed with water, and air-dried. The ground lead salt, divided into a number of portions, was then decomposed with the minimum quantity of 10 per cent methyl alcoholic hydrogen chloride, in which the pigment was soluble. The lead chloride was removed by filtration, and a little absolute ethyl alcohol added to the solution, after which it was placed in a shallow dish covered with filter paper.

The mixture slowly deposited the dark impure granular pigment on the bottom and sides of the dish. From time to time the solution was removed by decantation and allowed to stand until the separation of granular pigment appeared to be nearly complete, although the color of the final mother liquor still indicated the presence of an appreciable quantity of coloring matter. The precipitates were washed with a little absolute ethyl alcohol and ether, after which they were air-dried and converted into the picrate.

*Purification by Means of Picrate*—The crude pigment was pulverized in an agate mortar and dissolved in hot (60°) saturated aqueous picric acid. Insoluble gummy impurities were filtered off while hot, and the deep orange-red solution was kept in a cool place for 1 week. The crude picrate was recrystallized from half saturated picric acid solution. It was decomposed, 10 per cent methyl alcoholic hydrogen chloride being employed, and the parent

substance recovered by precipitation with ether. After being filtered and washed with ether to remove picric acid, the pigment was dried in a desiccator and ground. Subsequently the brown-red powder was dissolved in hot methyl alcohol and a little concentrated hydrochloric acid and absolute ethyl alcohol added. Allowed to evaporate slowly in a shallow dish as before, the solution deposited the coloring matter in much purer form, as shown by its bronze luster. It was reconverted into the picrate, a portion of which was prepared for analysis by several recrystallizations from hot water containing a slight excess of picric acid. As thus obtained the picrate formed deep brownish red slender prisms or needles (Fig. 1) which in mass exhibited a metallic luster and melted at 177.5–178.5°. Analysis<sup>2</sup> of the air-dried substance gave C 44.12, H 3.69, N 5.53, H<sub>2</sub>O 7.36. Chrysanthemin picrate, C<sub>27</sub>H<sub>23</sub>O<sub>18</sub>N<sub>3</sub>·3H<sub>2</sub>O, requires C 44.31, H 4.00, N 5.75, H<sub>2</sub>O 7.39. After drying the picrate in a high vacuum at 111°, analysis gave C 47.60, H 3.51, N 6.20. C<sub>27</sub>H<sub>23</sub>O<sub>18</sub>N<sub>3</sub> requires C 47.84, H 3.42, N 6.20. The picrate is easily soluble in alcohol, from which it is mainly precipitated with ether, slightly soluble in water, and much more soluble in hot water. It is partly decomposed in aqueous solution when heated above 70°.

The bulk of the crude picrate was reconverted as before into the chloride, which was repeatedly crystallized by dissolving in a minimum quantity of hot 0.01 per cent hydrochloric acid, filtering, and treating with 5 volumes of warm 3 per cent ethyl alcoholic hydrogen chloride. On cooling there occurred a beautiful glistening precipitate of chrysanthemin chloride having a golden bronze luster. The crystals were obtained as diamond-shaped platelets (Fig. 2) which under the microscope reflected a beautiful play of delicate colors. The precipitate was filtered with slight suction and washed with 7 per cent aqueous hydrochloric acid. The total final quantity of pure coloring matter so obtained amounted to 8.5 gm., air-dried. From the filtrate on hydrolysis with 20 per cent hydrochloric acid, a quantity of aglycone was obtained, which was used subsequently for a potassium hydroxide fusion.

*Chrysanthemin Chloride*, C<sub>21</sub>H<sub>21</sub>O<sub>11</sub>Cl—Chrysanthemin chloride, as obtained above, contains 1½ molecules of water of crystallization.

<sup>2</sup> Where more than one analysis was made, the averaged value is given throughout this paper.



It was dried over phosphorus pentoxide for 24 hours at 105–110° with a high vacuum oil pump and also for 4 hours at 111° in a very high vacuum obtained by means of the mercury vapor pump. Both methods yielded practically the same analytical results. Exposed to the air, anhydrous chrysanthemin chloride in micro-quantities reabsorbs its water of crystallization almost instantly. The substance was analyzed both in its air-dried state and in the anhydrous condition. The air-dried material gave C 49.77, H 4.80, Cl 7.10, H<sub>2</sub>O 4.88. Chrysanthemin chloride,  $C_{21}H_{21}O_{11}Cl \cdot 1\frac{1}{2}H_2O$ , requires C 49.25, H 4.73, Cl 6.93, H<sub>2</sub>O 5.28. The anhydrous material gave C 52.06, H 4.58, Cl 7.39.  $C_{21}H_{21}O_{11}Cl$  requires C 52.00, H 4.37, Cl 7.32.

In appearance and behavior with certain reagents the pigment agreed in every respect with chrysanthemin chloride isolated from the chrysanthemum (*Chrysanthemum indicum*, L.) by Willstätter and Bolton (16) and with a synthetic specimen kindly supplied by Professor Robert Robinson (13). It is easily soluble in water, forming a dark brownish red color, which on dilution assumes a bluish cast. It is almost as easily soluble in 0.01 per cent aqueous hydrochloric acid, but in general becomes less soluble as the concentration of acid is increased. The pigment is easily soluble in methyl alcohol, but only slightly in ethyl alcohol. Sodium carbonate imparts a violet or blue-violet color to its aqueous solution, whereas sodium hydroxide gives a pure blue color. In alcohol, ferric chloride yields a blue, which changes to violet color on dilution with water. Sodium acetate added to a 1 per cent aqueous hydrochloric acid solution of the pigment results in a violet-red color.

*Hydrolysis of Glucoside*—Chrysanthemin chloride, 0.5 gm., was dissolved in 18 cc. of hot water, and 17 cc. of concentrated hydrochloric acid were added. The solution was then boiled for 2 to 3 minutes, cooled immediately, and set aside overnight in an ice box. Crystals of the aglycone began to separate from the solution while boiling and when cooling. These were collected in a weighed Gooch crucible and washed with 7 per cent aqueous hydrochloric acid. Recovery of air-dried cyanidin chloride amounted to 67.31 per cent.  $C_{21}H_{21}O_{11}Cl \cdot 1\frac{1}{2}H_2O$  yields 66.57 per cent of cyanidin chloride,  $C_{15}H_{11}O_6Cl \cdot 1 H_2O$ .

The slightly red filtrate from the hydrolysis mixture was shaken

with amyl alcohol to remove traces of cyanidin chloride, then neutralized with lead carbonate. The lead chloride was filtered off, and the excess of lead removed by means of hydrogen sulfide. The solution was concentrated *in vacuo* and the sugar converted into its osazone by heating with phenylhydrazine and acetic acid. The yellow precipitate after recrystallization from 50 per cent alcohol was collected and dried. It melted at 204–205°, thus indicating that the hexose sugar in the purple corn pigment is glucose.

*Cyanidin Chloride*,  $C_{15}H_{11}O_6Cl$ —The hydrolytic product of chrysanthemin chloride crystallized from hot 20 per cent hydrochloric acid as long, slender brown-red needles (Fig. 3) which appeared under the microscope to be perfectly homogeneous and free of amorphous

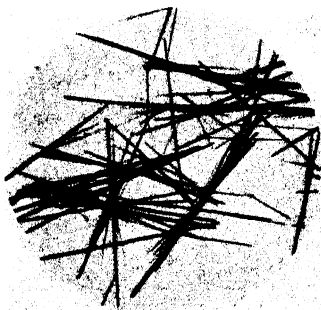


FIG. 1  
Chrysanthemin picrate

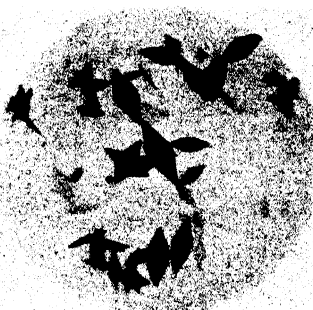


FIG. 2  
Chrysanthemin chloride

particles. It was recrystallized by dissolving in ethyl alcohol, adding one-half the quantity of 20 per cent aqueous hydrochloric acid, and allowing the alcohol to evaporate slowly from a shallow dish covered with filter paper. Cyanidin chloride crystallizes with 1 molecule of water of crystallization. On analysis, the air-dried sample gave C 52.77, H 4.27,  $H_2O$  5.55. Cyanidin chloride,  $C_{15}H_{11}O_6Cl \cdot H_2O$ , requires C 52.85, H 3.85,  $H_2O$  5.29. The anhydrous sample, dried in a high vacuum at 111°, gave C 55.53, H 3.73;  $Cl$  11.63.  $C_{15}H_{11}O_6Cl$  requires C 55.80, H 3.44,  $Cl$  10.99.

The identity of the aglycone with cyanidin chloride was further confirmed by determining the cleavage products in the usual way by potassium hydroxide fusion. The material was heated with potassium hydroxide and a very small amount of water to 200–220°

for 5 minutes, and then the temperature raised rapidly to 250°. The melt was dissolved in water, neutralized with hydrochloric acid, and shaken with ether. After the ethereal solution was evaporated, the residue was neutralized with sodium bicarbonate in aqueous solution and again shaken with ether. The sodium carbonate solution, from which the phenol was removed, was acidified and shaken out with ether to remove the acid portion. In each case the residue was dissolved in hot water and decolorized with blood charcoal. The clear solution was then evaporated until separation of the solid occurred on standing. The phenol proved to be phloroglucinol, identified by means of the characteristic red color imparted to a pine shaving, when mixed with

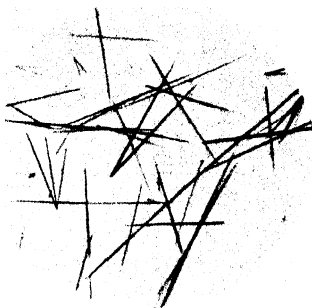


FIG. 3. Cyanidin chloride

hydrochloric acid. The acid proved to be protocatechuic acid, identified by its sublimate of catechol and by the green color exhibited with ferric chloride.

#### SUMMARY

1. Chrysanthemin chloride, an anthocyanin coloring matter, has been isolated in pure form and identified as such from purple-husked maize. The latter is one of a series of color types in maize whose heritable behavior has been determined by Emerson. Since factorial compositions have been assigned to this series of color types and it has been shown that flavonol and anthocyanidin pigments are involved as color characters, it goes without saying that this series affords ideal material for a chemical interpretation of the Mendelian factors involved. The pigments have now been worked

out from the brown-husked and purple-husked types. The evidence obtained thus far favors the possibility of a conversion of the flavonols to anthocyanidins by reduction of their corresponding homologous glucosides.

2. Chrysanthemin chloride was purified in the form of its picrate. The regenerated pigment on hydrolysis yields molecular proportions of cyanidin chloride and glucose.

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# A COMPARISON OF THEELIN PREPARED FROM STALLION URINE, HUMAN URINE, AND FROM THEELOL, WITH NOTES ON THE COLORIMETRIC ESTIMATION OF THEELIN AND THEELOL

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Zondek (1) has reported that the urine of the stallion contains a high concentration of estrogenic hormone. Deulofeu and Ferrari (2) and Häussler (3) have isolated from stallion urine a crystalline hormone apparently identical with theelin.

Since the urine of the pregnant mare yields a theelin crystalline material composed of a difficultly separable mixture of closely related substances, we were particularly interested in determining whether the isolation of pure theelin from stallion urine offers similar difficulties. Our results indicate that stallion urine in contrast to mare urine readily yields pure theelin.

Butenandt and Störmer (4) have described a  $\beta$  isomer of theelin obtained from mare urine and by dehydration of theelol, which differs mainly from the  $\alpha$  form in that it is approximately one-fifth as active biologically. In view of the recent report of Curtis, MacCorquodale, Thayer, and Doisy (5) regarding the great variation often encountered in the biological assays of estrogenic substances, the question of the existence of  $\alpha$  and  $\beta$  isomers can be answered only by parallel assays such as those reported by these workers for specimens of theelin obtained from different sources. We have confirmed the findings of Doisy's group (5) and consequently realized the necessity of making parallel chemical and biological studies on theelin specimens obtained from different sources in order to determine whether the theelin from stallion urine exists in an  $\alpha$  or  $\beta$  form. We have obtained no evidence for the existence of a less active  $\beta$  isomer in stallion urine but have been able to

obtain approximately 60 per cent of the total activity of the original urine as crystalline theelin, agreeing in chemical, physical, and biological properties with theelin from human urine. Our results are also in agreement with Doisy's in that we have failed to obtain evidence of a  $\beta$  form of theelin made from theelol by dehydration under various conditions.

#### EXPERIMENTAL

*Preparation of Theelin from Stallion Urine*—Representative specimens of urine from four stallions assayed biologically in spayed rats by the method of Kahnt and Doisy (6) showed variations of from practically 0 (2 year-old colt) to 38,000 rat units per liter.

The urine was covered with  $\frac{1}{3}$  volume of butanol, acidified with strong sulfuric acid to Congo red, and refluxed on the steam bath for 4 hours. Complete extraction of hormone was accomplished by two subsequent extractions in a shaking machine with butanol at room temperature. This procedure is effective and avoids difficulties due to foaming or troublesome emulsions. The combined butanol extracts were washed with 2 per cent  $\text{Na}_2\text{CO}_3$  and concentrated *in vacuo* under nitrogen to a brown oil. From this point, further fractionation was carried out by the method of Butenandt (7), which is briefly as follows:

The brown oil was distributed between 50 per cent methanol and petroleum ether, the part remaining in 50 per cent methanol being called *Hormoncharge II* by Butenandt. The dilute methanol-soluble fraction was distributed between 60 per cent ethanol and benzene; the benzene-soluble fraction contains theelin (*Hormoncharge III*). The 60 per cent ethanol-soluble fraction, in the case of human urine, contains theelol which is further purified by Butenandt by the same method as was used for the theelin fraction. We have been unable to find evidence of theelol in this fraction from stallion urine but for convenience we refer to this as the "theelol" fraction in Table II.

The benzene-soluble theelin fraction was hydrolyzed with dilute alcoholic HCl, extracted with ether, washed, and then extracted with *N* NaOH. The *N* NaOH extract was acidified and extracted with ether, yielding an oil rich in theelin (*Hormoncharge IV*). At this point we omitted the high vacuum distillation used by But-

enandt and proceeded directly to crystallization of the hormone from dilute alcohol. Stallion urine assaying 38,000 rat units per liter by this process yielded per liter 16 mg. of crystalline hormone, m.p. 246–248° uncorrected, which accounts for approximately 60 per cent of the biological activity of the original urine. Two crystallizations from alcohol yielded colorless crystals, m.p. 253–254.5° uncorrected.

*Analysis*— $C_{18}H_{22}O_2$ . Calculated. C 79.95, H 8.21  
Found. " 79.97, " 8.42

*Theelin by Dehydration of Theelol*—Theelol (m.p. 275–276° uncorrected,  $[\alpha]_{5461}^{25} = +71^\circ$  (in alcohol)) was intimately mixed with 40 parts of fused potassium bisulfate and sublimed at 180–200° at 0.002 to 0.010 mm. The sublimate recrystallized three times from alcohol yielded colorless crystals, m.p. 254.5–255.5° uncorrected.

*Analysis*— $C_{18}H_{22}O_2$ . Calculated. C 79.95, H 8.21  
Found. " 79.93, " 8.15

*Theelin from Human Urine*—Late pregnancy urine was acidified to Congo red and extracted with butanol at room temperature. The butanol extracts were washed with 2 per cent  $Na_2CO_3$  and concentrated to a brown oil. Further purification was accomplished by either the method of Doisy and Thayer (8) or of Bute-nandt and Hildebrandt (9). The crude crystalline material, purified by recrystallization from alcohol, yielded colorless crystals, m.p. 253.5–254.5° uncorrected.

*Analysis*— $C_{18}H_{22}O_2$ . Calculated. C 79.95, H 8.21  
Found. " 80.01, " 8.14

*Comparison of Theelin from Stallion Urine, Human Urine, and from Theelol*—Characteristic chemical properties, physical properties, and biological assays for these theelin specimens of different origin are summarized in Table I.

The bioassays were carried out by the method of Kahnt and Doisy (6), except that the rat unit has been taken as the dose producing a full squamous smear in 50 per cent of the injected rats. Injections were made in 0.5 per cent aqueous sodium carbonate containing 10 per cent alcohol. Each specimen of theelin has been assayed by injecting theelin of international standard as a



control in parallel groups of rats. The results summarized in Table I are interpreted as furnishing satisfactory proof of the identity of theelin prepared from stallion urine, human urine, and from theelol. Our results with theelin prepared by dehydration of theelol agree with those of Doisy's group (5) in that we failed to obtain evidence of the presence of the  $\beta$  hormone described by Butenandt and Störmer (4). The dehydration of theelol with potassium bisulfate has been carried out rapidly at 200° and slowly at 160–180°, and in no case have we obtained evidence of a less active  $\beta$  isomer of theelin.

TABLE I

*Comparison of Theelin from Stallion Urine, Human Urine, and from Theelol*

Source of theelin.....	International standard	Stallion urine	Human urine	Theelol
M.p. (360° thermometer, uncorrected), °C.....	252–253	253–254.5	253 –254.5	254.5–255.5
M.p. (short stem Anschütz, corrected), °C.....	258–259	259–260.5	259.5–260.5	260.5–261.5
$[\alpha]_{\text{D}}^{25}$ in ethyl alcohol....		+190	+191	+190
$[\alpha]_{\text{D}}^{25}$ “ “ “ “.....		+167	+166	+163
M.p. of benzoate, uncorrected, °C.....		214–216	216 –217.5	216 –217
Assay (Doisy method), rat units per mg. ....	1440	1310	1270	1310

*Notes on Colorimetric Method of Assay*—Kober (10) has described a colorimetric method for the quantitative estimation of estrogenic hormones. Cohen and Marrian (11) have recently described a modification of this method which apparently gives results showing excellent agreement with the biological method. During the past year we have been using a modification of the Kober test which is much simpler than that described by Cohen and Marrian but which is sufficiently accurate to be of great value in the isolation of the crystalline hormones from urine. Since the adoption of this method we have practically dispensed with the biological assay except in the case of very crude extracts where discoloration produced by impurities interferes with the test.

*Method*—The method as originally described by Kober (10)

has been slightly modified. The phenolsulfonic acid reagent is easily prepared by heating a mixture of equal weights of phenol and concentrated sulfuric acid at 110–120° for 15 minutes. The mixture is cooled and diluted with 2 volumes of concentrated  $\text{H}_2\text{SO}_4$ .

Into a small fine tipped centrifuge tube introduce 0.1 cc. of an alcohol solution containing 0.005 mg. of standard theelin. Introduce a small glass bead to prevent bumping and remove the solvent on a water bath *in vacuo*. Into similar tubes introduce measured amounts of the unknown hormone solution estimated to contain 0.005 mg. of theelin, and remove the solvent. Add 0.2 cc. of phenolsulfonic acid reagent to each tube and mix by shaking. The glass bead facilitates this mixing. Heat in boiling water for 3 minutes, shaking the tubes once or twice to assure contact between the theelin and the reagent. A yellow color develops rapidly with theelin, more slowly with theelol. Cool in tap water for about 1 minute. Add 0.2 cc. of water to each tube, mix, and heat in a glycerol bath at 125° for 2 minutes. A red color develops. Cool in tap water for 1 minute, add 0.6 cc. of water to each tube, and mix. The colors are compared against a white background. In this way, by using a series of tubes, the volume of unknown solution containing the chromogenic equivalent of 0.005 mg. of standard theelin can be directly determined. In the case of the more purified and crystalline fractions the errors of reading the colors can be reduced by using a colorimeter.

*Results*—To illustrate the value of this colorimetric method in following the hormone concentrations of the various urine extracts at widely different stages of purity, we have summarized in Table II our results with extracts from one lot of human urine and from two lots of stallion urine, one of which was active and the other inactive. The stages of purification given in Table II refer to the *Hormoncharge* numbers of Butenandt (7) as defined above under "Preparation of theelin from stallion urine." The results show that the color equivalents closely follow the active fractions with no more than moderate losses between the most crude stage at which the colorimetric method is first applicable and the highly purified Stage IV at which the hormones can usually be crystallized from alcohol. It will be noted that for each fraction listed in Table II, the color equivalent is finally confirmed by biological

assay or actual isolation of the crystalline hormones in convincing amounts. In the case of the biologically inactive lot of stallion urine, Lot 13, the color assays at exactly analogous stages of purification showed no chromogenic substance to be present. In this case not a trace of crystalline theelin could be isolated from the

TABLE II  
*Colorimetric Assay Applied to Urine Fractions*

Urine extracted	Fraction	Stage of purification (Butenandt)	Total solids	Color equiv- alent	Remarks
			<i>gm.</i>	<i>mg. theelin</i>	
Lot 58. 418 liters human urine	Theelin + theelol	II	24.4	940	Divided into 2 fractions below
	Theelin	III	9.5	93	Biological as- say, 94,000 rat units
	"	IV	0.47	94	
	Theelol	III	13.0	930	
	"	IV	1.28	855	
	"	Crystals, m.p. 269-271°	0.745	745	
Lot 120. 2.6 liters stal- lion urine	Theelin + "theelol"	II	12.6	100	Biological as- say, 99,000 rat units
	Theelin	III	1.37	105	
	"	IV	0.36	75	
		Crystals, m.p. 246-248°	0.041	41	
Lot 13. 113 liters stal- lion urine (2 year-old colt)	Theelin + "theelol"	II	150	0	Biological as- say, inac- tive
	Theelin	III	37.2	0	
	"	IV	3.45	0	No crystals
	"Theelol"	III	65	0	
	"	IV	7.8	0	" "

purified fractions representing 113 liters of stallion urine. Thus we conclude that the colorimetric method is capable of furnishing a rapid estimate of the theelin or theelol content of active urine fractions and that lack of color development in any fraction constitutes valid evidence of the absence of these hormones.

*Limitations of Colorimetric Method*—Certain precautions in the use and interpretation of the colorimetric assay are essential in order to avoid serious error. Pure theelin and theelol show identical color equivalents although theelol is much less active than theelin in the adult spayed rat. Consequently, we cannot expect by the colorimetric method to measure the biological potency of unknown mixtures of theelin and theelol with any degree of accuracy. The color method fails when applied directly to the urine or the very crude extracts prepared from it. This failure is due to impurities which char or develop yellow or brown colors, masking the characteristic red produced by theelin or theelol. The use of the Lovibond tintometer as described by Cohen and Marrian (11) will undoubtedly be of help in cases where the discoloration is not too great. Usually satisfactory results are obtained in extracts having a theelin to impurities ratio of 1:20, although very often good results have been obtained in extracts with this ratio of only 1:100. The colorimetric method is at its best when used in evaluating the potency of unknown solutions of pure theelin or theelol. Although theelin and theelol give quantitatively the same end colors, the development of this color is sufficiently different in the two cases to permit the identification of pure solutions of either hormone. This difference depends upon the rate of development of the yellow color during the first heating at 100°. With theelin the yellow color develops strongly within a few seconds, whereas with theelol it requires 2 to 3 minutes for the maximum color to develop. Finally it should be mentioned that inactive substances are capable of giving a characteristic theelin color reaction by this method. For example, the residues remaining in the retort after dehydration of theelol in a high vacuum with potassium bisulfate have been found to give a strong color reaction although neither theelin nor theelol could be isolated from them. In this case the color is obviously due to decomposition products. Also, Kober (10) reports that anthrol gives this color reaction. The colorimetric behavior of the  $\delta$  follicular hormone of Schwenk and Hildebrandt (12) and of equilin, equilenine, and hippuline obtained from mare urine by Girard *et al.* (13) remain for future investigation.

#### SUMMARY

The preparation of pure theelin from stallion urine is described. Comparative data on the chemical, physical, and physiological

characteristics are offered to prove the identity of stallion theelin with that obtained from human urine and from theelol.

For the preparation of pure theelin by extraction methods; stallion urine is a superior source to mare urine because it yields theelin in a readily purified form relatively free from the closely related crystalline impurities which interfere in the case of mare urine.

Results obtained with a colorimetric method indicate the value of this method for the rapid quantitative estimation of theelin and theelol in pure solutions and in semipurified urine extracts. The limitations of the method are discussed.

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# ANALYSIS OF ALBUMIN AND GLOBULIN IN BIOLOGICAL FLUIDS BY THE QUANTITATIVE PRECIPITIN METHOD

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For several years investigators have been engaged in studies of the permeability of the capillary wall for protein and have attempted to determine the relative amounts of albumin and globulin in serum, lymph, and edema fluid. Howe's method (1) of salting out the globulin with sodium sulfate, although used by several authors (2-4) in the fractionation of the proteins of lymph, is unsatisfactory when the protein content is low and cannot be applied when the volume available for analysis is very small. Howe discusses the effect of the dilution of plasma on the accuracy of the separation of albumin and globulin; an examination of Table I in his original article shows that the procedure may result in a loss of albumin, the error sometimes being as high as 10 per cent. Errors of this magnitude cause wide fluctuations of the albumin to globulin ratio (3). In this laboratory the reliability of Howe's method for fractionating such fluids has been tested by analyzing normal dog serum and dilutions of the same serum with 0.90 per cent saline to simulate the concentrations of protein found in lymph. Three normal dog sera, selected at random, and dilutions of the same sera ranging from 0.25 per cent to 2.0 per cent of total protein, were analyzed. In Table I the determined results are compared with the calculated values. In each case precipitation of the globulin in the diluted serum was more complete than in the original serum and resulted in low values for albumin. That the discrepancy is greater than the error of the micro-Kjeldahl method for determining nitrogen is shown by the analysis of

the total protein content of the dilutions of Sera 321 and 322. The wide fluctuation in the albumin to globulin ratio resulting from this loss of albumin is also shown.

The development of the precipitin reaction as a quantitative method for the estimation of small quantities of antigens and haptens (5, 6) suggested that immunological methods might be useful in determining small amounts of albumin and globulin with increased accuracy. It was assumed as a working hypothesis that

TABLE I

*Comparison of Determined Values of Albumin and Globulin in Diluted Sera with Calculated Results. Analyses by Howe's Method*

The results are expressed in gm. per 100 cc.

Serum No.	Dilution	Albumin		Globulin		Total protein		Albumin Globulin	
		Deter- mined	Calcu- lated	Deter- mined	Calcu- lated	Deter- mined	Calcu- lated	Deter- mined	Calcu- lated
367	Undiluted	3.79		2.56		6.35		1.5	
367	1:6	0.53	0.63	0.53	0.43		1.06	1.0	1.5
367	1:12	0.24	0.32	0.29	0.21		0.53	0.8	1.5
367	1:24	0.12	0.16	0.15	0.11		0.27	0.8	1.5
321	Undiluted	3.71		2.67		6.38		1.4	
321	1:3	1.13	1.24	1.02	0.89	2.15	2.13	1.1	1.4
321	1:6	0.57	0.62	0.49	0.44	1.06	1.06	1.2	1.4
321	1:12	0.25	0.31	0.28	0.22	0.53	0.53	0.9	1.4
322	Undiluted	2.46		4.82		7.28		0.5	
322	1:3	0.73	0.82	1.72	1.61	2.45	2.43	0.4	0.5
322	1:6	0.33	0.41	0.88	0.80	1.21	1.21	0.4	0.5
322	1:12	0.17	0.21	0.45	0.40	0.62	0.61	0.4	0.5

These sera were also analyzed by the precipitin method at dilutions of 1:200 or 1:300. A comparison of the results is shown in Table II.

the albumin and globulin fractions of the body fluids have the same immunological specificity as the corresponding fractions of the serum. The problem is complicated by the fact that serum protein contains five immunologically distinct fractions (7). Two of these, pseudoglobulin and euglobulin, are precipitated at half saturation with ammonium sulfate and therefore make up the globulin fraction as ordinarily determined. The filtrate from the precipitated globulin, usually considered albumin, contains a mucoprotein and two albumins that differ immunologically.

Application of the precipitin method to the analysis of protein fractions in biological fluids involves: (1) the preparation of antisera that will precipitate all of the antigenic components of one fraction without marked cross precipitation of other fractions; (2) the standardization of these antisera with known quantities of serum albumin and serum globulin; (3) the estimation of albumin and globulin in unknown biological fluids by comparison with the standardization curves of the antisera.

*Preparation of Antigens*—Albumin and globulin fractions were prepared from normal dog serum. The globulin was precipitated at half saturation with ammonium sulfate, washed, dissolved in saline, and reprecipitated twice to remove traces of albumin.

Since the filtrate from the original precipitation of the globulin contained all of the antigenic components of serum except euglobulin and pseudoglobulin, it was selected as most nearly representing the albumin fraction as ordinarily determined. Both preparations were dialyzed free from ammonium sulfate and analyzed for nitrogen and non-protein nitrogen content by a modification of the Pregl micro-Kjeldahl method (8).

*Preparation of Antisera*—Alum-precipitated suspensions (9) of the albumin and globulin fractions were prepared and injected into the ear veins of rabbits. A course of eighteen injections resulted in a total administration of approximately 11.4 mg. of antigen per rabbit. The rabbits showing a sufficiently high antibody titer in test bleedings were bled from the heart and the antisera to each antigen pooled. Both antisera were sterilized by filtering through a Berkefeld N candle; merthiolate was added to a total concentration of 0.01 per cent; and the sera were stored in small rubber-stoppered bottles in the ice box.

*Standardization of Antisera*—The pooled antisera showed a small amount of precipitation when set up against the heterologous antigen. No attempt was made to free them from this cross reacting material because each of the antigens was known to contain traces of the other fraction, and absorbing out the heterologous antibodies with these impure antigens would remove an important fraction of the homologous antibodies. Since all of the biological fluids to be analyzed contained both albumin and globulin, the error due to cross precipitation could be corrected by standardizing the antisera against dilutions of normal dog serum containing



known amounts of albumin and globulin. The method of standardization was essentially that of Heidelberger and Kendall (5).

The dog serum used for standardization contained 33 mg. of albumin and 30 mg. of globulin per cc., as determined by Howe's method. Dilutions of this serum were made with sterile saline so that concentrations ranging from 0.1 to 0.4 mg. of albumin per cc. and of 0.1 to 0.4 mg. of globulin per cc. were available. Duplicate samples of the diluted antigens were measured out into sterile Wassermann tubes, the volume made up to 2 cc. with saline, and 1 cc. of the homologous antiserum added to each tube. Blanks were set up in duplicate with 2 cc. of saline and 1 cc. of antiserum. Calibrated pipettes were used throughout but were not sterilized. The contents of each tube were thoroughly mixed by carefully stroking the tube and giving it a swirling motion. The tubes were plugged with rubber stoppers, held at 37° for 2 hours, and placed in the ice box overnight.

The following morning the tubes were centrifuged for 10 minutes in a small angle centrifuge which was placed in a refrigerator regulated to maintain a temperature of 4°. The supernatant fluid<sup>1</sup> was carefully decanted into a small test-tube without loss of precipitate and the tube was inverted for a few minutes on a towel to drain. Excess fluid was wiped off and the tubes were returned to a test-tube rack imbedded in ice and water. Each precipitate was dispersed in 2 cc. of ice-cold saline, allowed to stand for 30 minutes in ice water, centrifuged, and carefully drained. After a second washing with 2 cc. of saline in a similar manner, the precipitate was dissolved in 5 drops of normal sodium hydroxide solution and rinsed quantitatively into a micro-Kjeldahl flask. Duplicate determinations of the total nitrogen in the precipitates showed a maximum variation of 3.4 per cent. In Chart I the amount of nitrogen precipitated is plotted against the quantity of antigen used.

*Analysis of Biological Fluids*—Precipitin analyses were carried out on serum, lymph, edema fluid, and ascitic fluid from dogs. Total nitrogen was first determined on a small sample of the fluid to estimate the approximate albumin and globulin concentrations. Dilutions of the fluid containing about 0.30 mg. of albumin and

<sup>1</sup> The supernatant fluid was tested for excess of antiserum by the addition of a small quantity of antigen.

0.25 mg. of globulin were set up in duplicate with the two antisera, the technique described in the standardization of the antisera<sup>2</sup> being used. The amount of globulin and albumin in the sample analyzed was determined from the precipitated nitrogen by comparison with the standardization curves in Chart I. The supernatant fluids were always tested for excess antibody by the addition of

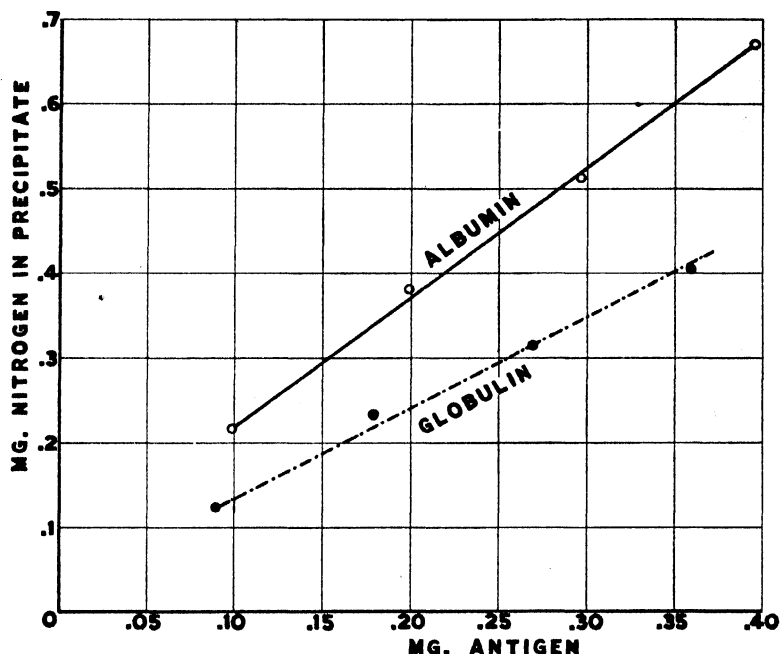


CHART I. Serum standardization

antigen. The method allows for a considerable variation in the quantity of albumin and globulin present in the analyses, but the greatest accuracy is obtained at the levels indicated.

Twenty-five analyses on sera from sixteen dogs were carried out in duplicate, the precipitin method being compared with Howe's method. In the latter the average difference for duplicate

<sup>2</sup> Most normal sera were analyzed at dilutions ranging from 1:200 to 1:300, lymphs from 1:25 to 1:50, and edema fluids from no dilution to 1:25.

determinations of albumin was 2.2 per cent, with a maximum difference of 6.4 per cent. For the precipitin analyses the average

TABLE II

*Analysis of Serum from Dogs. Comparison of Howe's Method and Precipitin Method*

Dog No.	Method	Albumin	Globulin	Total protein	Albumin Globulin	Remarks
		<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>		
189	Howe	3.89	2.07	5.96	1.9	Normal
	Precipitin	3.77	2.47	6.24	1.5	
191	Howe	2.42	2.16	4.58	1.1	"
	Precipitin	2.21	2.32	4.53	1.0	
207	Howe	3.24	1.81	5.05	1.8	"
	Precipitin	3.27	2.43	5.70	1.4	
246	Howe	3.86	2.49	6.35	1.6	"
	Precipitin	3.60	3.19	6.79	1.1	
293	Howe	2.94	2.90	5.84	1.0	"
	Precipitin	2.24	2.79	5.03	0.8	
296	Howe	3.37	2.62	5.99	1.3	"
	Precipitin	3.94	2.47	6.41	1.6	
321	Howe	3.71	2.67	6.38	1.4	"
	Precipitin	3.68	2.76	6.44	1.3	
322	Howe	2.46	4.82	7.28	0.5	"
	Precipitin	2.23	4.76	6.99	0.5	
336	Howe	4.26	1.91	6.17	2.2	"
	Precipitin	4.19	1.83	6.02	2.3	
347	Howe	2.98	2.64	5.62	1.0	"
	Precipitin	2.84	2.78	5.62	1.0	
367	Howe	3.79	2.56	6.35	1.5	"
	Precipitin	3.70	2.54	6.24	1.5	
387	Howe	2.96	1.88	4.84	1.6	"
	Precipitin	3.17	1.70	4.87	1.9	
190	Howe	1.37	3.05	4.42	0.5	Edematous
	Precipitin	1.03	3.43	4.43	0.3	
205	Howe	1.95	3.53	5.48	0.6	"
	Precipitin	1.47	4.37	5.84	0.3	
206	Howe	0.99	3.04	4.03	0.3	"
	Precipitin	0.74	3.46	4.20	0.2	
840	Howe	1.29	3.32	4.61	0.4	"
	Precipitin	1.07	3.50	4.57	0.3	

difference for duplicate determinations of both albumin and globulin was 2.9 per cent, with a maximum difference of 7.1 per cent.

The data resulting from the analysis of a single serum from each dog are presented in Table II.

Thirty-one samples of subcutaneous lymph obtained at various intervals from thirteen dogs were analyzed in duplicate by both methods. Table III summarizes the results of a single lymph from each dog. The duplicate determinations of albumin by Howe's method showed an average difference of 9.7 per cent, with a maximum difference of 32.0 per cent, and by the precipitin method an average difference of 2.6 per cent, with a maximum difference of 7.5 per cent. The duplicate analyses of globulin by the precipitin method gave essentially the same variations, an average difference of 2.0 per cent, with a maximum difference of 5.7 per cent.<sup>3</sup>

In Table IV are summarized the results obtained on the analyses of edema and ascitic fluids to date. The series is small as few experimental animals were available for this particular study. Agreement of the total protein figures is consistent, but in this group fractionation of the proteins by salting out was not possible.

It was pointed out previously that serum albumin and serum globulin cannot be looked upon as single antigens but that each contains two or more immunologically distinct fractions. Because the fractions of a multiple antigen may vary in their ability to evoke antibody formation and because the distribution of these fractions in individual animals will not conform exactly with that in the antigens used for preparing antisera, the precipitin method was not expected to yield accurate values for the total amounts of albumin and globulin in the sera of normal dogs. The fairly close agreement which was obtained for albumin, globulin, and total protein values, therefore, came as a surprise. In five dogs (Dogs 246, 296, 205, 293, and 207) there is some discrepancy in the total protein figure; in four dogs (Nos. 190, 296, 205, and 293) the albumin determinations show variation; and in six dogs (Nos. 190, 206, 189, 246, 205, and 207) globulin values do not agree. Although the reason for these discrepancies is not known, they suggest some difference in the character of the protein fractions in different dogs, particularly as repeated determinations on sera drawn from the same dog at different times show consistent

<sup>3</sup> It is seen that the percentage error by Howe's method increases rapidly when fluids low in protein are to be analyzed, a factor which does not affect the accuracy of measurement by the precipitin method.

variations. Some of the differences might be obviated by using pooled sera instead of a single serum in the preparation of the antigens. It was also surprising to find that in other body fluids the total protein figures obtained in the precipitin method by the

TABLE III

*Analysis of Subcutaneous Lymph from Dogs. Comparison of Howe's Method and Precipitin Method*

Dog No.	Method	Albumin	Globulin	Total protein	Albumin Globulin	Remarks
		gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.		
189	Howe	1.28	0.69	1.97	1.9	Normal
	Precipitin	1.48	0.66	2.14	2.2	
191	Howe	0.49	0.54	1.03	0.9	"
	Precipitin	0.46	0.59	1.05	0.8	
192	Howe	0.36	0.22	0.58	1.6	"
	Precipitin	0.40	0.19	0.59	2.1	
207	Howe	0.61	0.33	0.94	1.9	"
	Precipitin	0.74	0.35	1.09	2.1	
246	Howe	0.85	0.38	1.23	2.2	"
	Precipitin	0.88	0.45	1.33	2.0	
367	Howe	0.97	0.61	1.58	1.6	"
	Precipitin	1.08	0.56	1.64	1.9	
387	Howe	0.74	0.37	1.11	2.0	"
	Precipitin	0.81	0.25	1.06	3.2	
190	Howe	0.14	0.35	0.49	0.4	Edematous
	Precipitin	0.13	0.36	0.49	0.4	
205	Howe	0.56	1.15	1.71	0.5	"
	Precipitin	0.42	1.34	1.76	0.3	
206	Howe	0.21	0.77	0.98	0.3	"
	Precipitin	0.20	0.79	0.99	0.3	
293	Howe	0.20	0.36	0.56	0.6	"
	Precipitin	0.16	0.33	0.49	0.5	
336	Howe	0.19	0.46	0.65	0.4	"
	Precipitin	0.16	0.41	0.57	0.4	
840	Howe	0.15	0.48	0.63	0.3	"
	Precipitin	0.19	0.44	0.63	0.4	

addition of the albumin and globulin fractions compared so favorably with the results of direct chemical analyses. This suggests that in the dog under the above experimental conditions all the protein present in the various body fluids has the same immunological specificity as the serum proteins.

In the determination of albumin to globulin ratios of fluids of low protein content the precipitin method has particular validity. It has already been shown that the accuracy of the precipitin method increases as the total protein content decreases, since the total protein is obtained by multiplying the amount found in the sample tested by the dilution factor, while the error of Howe's method rises progressively and rapidly. Moreover, since in

TABLE IV

*Analyses of Edema Fluids and Ascitic Fluids. Comparison of Direct Chemical Analysis and Precipitin Method.*

Dog No.	Type of fluid	Method	Albumin	Globulin	Total protein	Albumin Globulin
			<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	
190	Edema	Kjeldahl			0.113	
		Precipitin	0.055	0.081	0.136	0.7
190*	"	Kjeldahl			0.100	
		Precipitin	0.051	0.076	0.127	0.7
206	"	Kjeldahl			0.290	
		Precipitin	0.073	0.250	0.323	0.3
207	"	Kjeldahl			0.079	
		Precipitin	0.030	0.054	0.084	0.6
207	Ascitic	Kjeldahl			0.035	
		Precipitin	0.015	0.025	0.040	0.6
293	Edema	Kjeldahl			0.72	
		Precipitin	0.37	0.36	0.73	1.0
336	"	Kjeldahl			0.225	
		Precipitin	0.045	0.149	0.194	0.3
336	"	Kjeldahl			0.217	
		Precipitin	0.058	0.140	0.198	0.4

Howe's method globulin is determined by difference, an error in the numerator of the ratio becomes necessarily paired with an error of opposite sign in the denominator, so that neutralization of the two errors cannot possibly take place. The error of the quotient is smaller in the precipitin method by reason of the fact that albumin and globulin are analyzed separately.

Albumin to globulin ratios determined on fluids taken simultaneously from the same animal should be strictly comparable even if they may not be compared with ratios obtained by salting

out the proteins. When the precipitin method was used to analyze serum, lymph, edema fluid, and ascitic fluid taken on the same day from various dogs, the albumin to globulin ratios of lymph, edema fluid, and ascitic fluid were consistently higher than the albumin to globulin ratio of the corresponding serum. This relation is

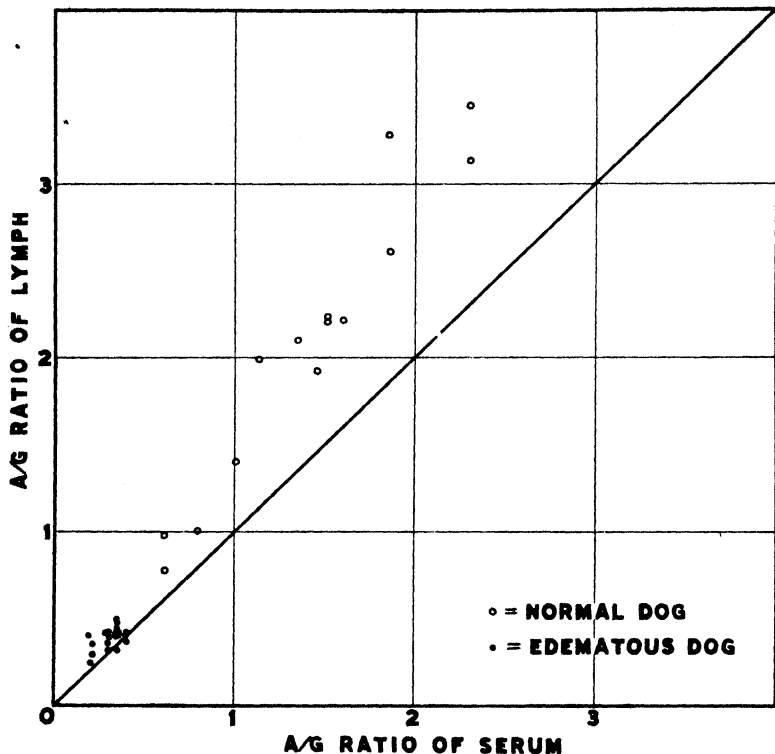


CHART II. Comparison of the albumin to globulin ratios of serum and lymph of dogs. The diagonal line represents identity of the ratios.

shown graphically in Chart II. When Howe's method was used, such consistent results were not obtained. The increase in the albumin to globulin ratio of the various fluids which have passed through a capillary wall as compared with the albumin to globulin ratio of the corresponding serum suggests a selective permeability for albumin.

The precipitin method promises to be of particular value in the fractional analysis of protein in fluids of low protein content. It may also be used for the determination of comparative albumin to globulin ratios in fluids of higher protein content when only small quantities are available for analysis. Total protein in fluids of low protein content may be estimated accurately by adding the albumin and globulin as determined by this method.

The authors wish to thank Dr. M. Heidelberger and Dr. A. A. Weech for their interest and cooperation.

#### SUMMARY

1. A new method is presented for the differential analysis of albumin and globulin in fluids of low protein content. The general agreement with the total protein determined by chemical analysis affords further confirmation of the accuracy of the absolute method of precipitin determinations.

2. Data obtained by this method are presented which suggest a selective permeability of the capillary wall for albumin.

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## THE CARBON MONOXIDE CAPACITY, IRON, AND TOTAL NITROGEN OF DOG HEMOGLOBIN

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Since 1866 there have been not less than twenty-six published investigations of the oxygen or carbon monoxide capacity of mammalian hemoglobins, exclusive of miscellaneous iron and molecular weight determinations from which the capacity values may be calculated.<sup>1</sup> All investigators except Morrison (2) have determined the hemoglobin concentration by indirect methods, spectrophotometric and iron analyses having been most frequently employed. In only one of the investigations (2) were the precision manometric methods of Van Slyke and his associates employed and crystallized hemoglobin was used only in an occasional instance. Of the twenty-five final reports, in only three did workers use sufficiently precise methods to obtain consistent results. Their range of values in cc. of carbon monoxide per gm. of hemoglobin is as follows: Hüfner (3) obtained one value of 1.30 and five values from 1.34 to 1.36; Saint-Martin (4), 1.33 to 1.35; and Butterfield (5), 1.30 to 1.35. Peters (6), using the Barcroft and Roberts (7) method, found that from 383 to 400 cc. of oxygen (average 393 cc.) combined with the hemoglobin containing 1 gm. of iron, but he did not have sufficient data to calculate the oxygen capacity per gm. of hemoglobin. Accurate iron determinations (Hüfner (3)) indicate that the accepted capacity value of 1.34 cc. of oxygen or carbon monoxide per gm. of hemoglobin is somewhat too low. Hüfner, Saint-Martin, and Butterfield determined the hemoglobin concentrations of their solutions spectrophotometrically. The validity of such measurements depends finally upon

<sup>1</sup> See the table in Wertheimer's (1) paper and preliminary report by Morrison (2).

the purity of the hemoglobin or hemoglobin derivative employed in calibration of the spectrophotometer. Recent values obtained by Kennedy (8) and Winegarden and Borsook (9) for the extinction coefficients of carboxyhemoglobin and oxyhemoglobin are higher than those used by earlier workers and indicate that earlier values for the oxygen and carbon monoxide capacity are significantly low.

TABLE I  
*Total Nitrogen of Hemoglobins*

Species	Observer	Total N <i>per cent</i>
Dog	Jaquet (12)	15.98
"	Schmidt (13)	16.33
"	Hoppe-Seyler (14)	16.17
"	Block (15)	16.40
"	Morrison and Hisey	16.49
Ox	Hüfner, <i>cf.</i> (16)	17.70
"	Roche, Roche, Adair, and Adair (16)	16.75
Horse	Zinnoffsky (17)	17.94
"	Jaquet (12)	17.94
"	Schultz (18)	17.33
"	Nencki (19)	17.06
"	Kossel, <i>cf.</i> (20)	17.31
"	Otto, <i>cf.</i> (20)	17.28
"	Bucheler, <i>cf.</i> (20)	17.61
"	Hüfner (20)	17.61
"	Jorpes (21)	16.80
"	Block (15)	16.70
Human	Haurowitz (22)	17.0

Precise values for physically dissolved gases in hemoglobin solutions have only recently been obtained by Van Slyke and associates (10, 11). Earlier workers applied too low corrections for physically dissolved gases. It is interesting to note that this error was nearly compensated by the low extinction coefficient employed in determining hemoglobin concentrations.

Published values for total nitrogen of the hemoglobins vary widely, as shown in Table I.

### *Methods*

Fresh human, ox, or dog blood (defibrinated or oxalated) was centrifuged and the serum or plasma removed by a pipette attached

to suction. The cells were washed four to five times with 0.9 per cent sodium chloride solution. Purified hemoglobin solutions were prepared from the washed cells by three different procedures: (1) laking the cells with distilled water without further purification; (2) further purification by the alumina cream method of Marshall and Welker (23), and subsequent dialysis in cellulose casing until chloride-free; (3) crystallization of the hemoglobin.

Crystalline hemoglobin was prepared from dog blood by either the method of Heidelberger (24) or that of Ferry and Green (25). As a supercentrifuge was not available, the Ferry and Green procedure was modified by washing the cells four times in a centrifuge of large capacity and laking the cells with toluene. The remainder of the procedure was essentially that of the authors. By either method we obtained what appeared to be complete crystallization within a short time. The crystals were separated by centrifuging in chilled cups and the stroma and toluene were removed by suction. After washing with ice-cold distilled water (four to five times) in the centrifuge until all visible traces of stroma and toluene were removed, the crystals were dissolved and recrystallized by the method of Ferry and Green (25). The crystals obtained from this second crystallization were washed four to six times in the same manner as before. After the final washing they were dissolved in distilled water and aliquots of these solutions were analyzed immediately.

All analyses involving the activity of the hemoglobin were completed within 18 hours from the time the blood was drawn from the animal, and usually within a much shorter period. The only exception to this routine was in the case of Samples 1 to 3 (Table III) which were crystallized as carbon monoxide hemoglobin and dialyzed in cellulose casing against distilled water, saturated with a mixture of carbon monoxide and carbon dioxide gases, until chloride-free. All operations of crystallization and dialysis were carried out at as low a temperature as possible. It may be noted that the three dialyzed samples retained more base than the washed samples, as indicated by greater solubility of the former.

Carbon monoxide capacity and capacity after reduction were determined upon 5 cc. aliquots of the hemoglobin solutions by the methods of Van Slyke and Hiller (26) in a manometric apparatus. The distilled water was reduced by 3 cc., since there were no cells

to lake. Water from a constant temperature bath continually flowed through the jacket of the Van Slyke-Neill pipette.

The hemoglobin concentration was determined by weighing the residue from aliquots of the solutions after evaporating and drying to constant weight at 105°. In the case of the purified (Procedure 2) solutions we have also precipitated the hemoglobin in aliquots by acetone and after washing with acetone filtered on Jena glass crucibles and dried at 105°. Weighed samples of the dried hemoglobin and aliquots of the original solutions were analyzed for total nitrogen by the Kjeldahl method. The values were the same in either case. In addition we have pipetted aliquots of these solutions into a number of Kjeldahl flasks. Some of these flasks were placed in an oven and the contents dried at 105° for 48 hours, before the sample was digested. The samples in the other flasks were digested immediately. Again, the results were the same. This would demonstrate that whatever change takes place on drying hemoglobin does not involve a loss of nitrogen.

The acid and alkali used in the total nitrogen determinations were standardized directly or indirectly with two different lots of constant boiling hydrochloric acid, with sodium carbonate, and as a further check the ammonia from weighed samples of recrystallized ammonium sulfate was distilled and quantitatively recovered. Hemoglobin Samples 2 and 3 were also analyzed independently<sup>2</sup> by another worker in the department with his own solutions, and his results were in perfect agreement with ours.

For the iron determinations we have used the titanous reduction method with a technique similar to that of Klumpp (27), but have determined the end-point with a potentiometer.

The weights used were calibrated against a weight certified by the United States Bureau of Standards and all volumetric apparatus, including the Van Slyke-Neill pipette, was calibrated with these weights. Mercury was used in the calibration of the smaller, and boiled out distilled water for the larger, apparatus.

#### EXPERIMENTAL

In Table II are summarized the carbon monoxide capacity to iron content ratios of seventeen samples of human hemoglobin

<sup>2</sup> We are indebted to Dr. E. P. Laug for these independent analyses.

prepared by Procedure 1. The average value of  $0.400 \pm 0.005$  cc. compares with the theoretical value of 0.401 cc. which assumes a ratio of 1 atom of iron to 1 molecule of carbon monoxide.

On seven different samples of purified dialyzed human hemoglobin (Procedure 2) we have obtained a carbon monoxide capacity ranging from 1.29 to 1.34 cc. per gm. One sample of ox blood purified by this procedure gave a value of 1.34 cc. per gm. Hemoglobin precipitated by alcohol or acetone from similar purified preparations and dried at  $105^\circ$  gave values for iron of 0.310 to 0.339 per cent. We then abandoned this mode of purification as impractical for our work.

TABLE II  
*Carbon Monoxide Capacity and Iron Content of Human Hemoglobin*

Sample No.	CO capacity	Iron	Cc. CO Mg. Fe	Sample No.	CO capacity	Iron	Cc. CO Mg. Fe
	<i>vol. per cent</i>	<i>mg. per cent</i>			<i>vol. per cent</i>	<i>mg. per cent</i>	
1	17.58	44.00	0.400	10	16.35	40.98	0.399
2	12.20	30.38	0.401	11	15.28	38.40	0.398
3	18.05	45.23	0.400	12	15.80	39.70	0.398
4	12.57	31.00	0.405	13	19.74	49.33	0.400
5	22.07	55.00	0.401	14	20.17	50.47	0.400
6	20.10	50.12	0.401	15	13.53	33.63	0.402
7	11.72	29.34	0.400	16	11.61	29.24	0.397
8	20.45	50.56	0.404	17	14.56	36.22	0.402
9	20.88	52.45	0.398				
				Average.	16.63	41.53	0.400

On three samples of recrystallized dog hemoglobin dried at  $105^\circ$  we have obtained the following results.

Hb gm.	Fe mg.	Fe per cent	CO cc.
3.4023 gave	11.56	$= 0.340$	equivalent to 1.363
5.0664	17.10	0.338	1.355
3.9516	13.32	0.337	1.351

In Table III are given the results of direct determination of carbon monoxide capacity and total nitrogen values of recrystallized dog hemoglobin. Each of the ten samples was analyzed for active capacity and capacity after reduction. The values were identical within the limits of experimental error except for Samples 2 and 3

whose values are reported as capacity after reduction. The nitrogen values represent averages of three or more determinations.

## DISCUSSION

Our results for the carbon monoxide capacity<sup>3</sup> of hemoglobin are slightly higher than the averages reported by other workers. These values more nearly represent the minimal capacity values than do the average values of other workers for the following reasons. (1) Our values were determined by methods which eliminate any correction for physically dissolved carbon monoxide and purity of a primary standard. (2) The highest consistent

TABLE III  
*Carbon Monoxide Capacity and Total Nitrogen of Dog Hemoglobin*

Sample No.	CO capacity	Hb	$\frac{\text{Cc. CO}}{\text{Gm. Hb}}$	Hb	$\frac{\text{Cc. CO}}{\text{Gm. N}}$
	<i>vol. per cent</i>	<i>gm. per cent</i>		<i>per cent N</i>	
1	8.56	6.337	1.351	16.57	8.15
2	7.89	5.863	1.346	16.49	8.16
3	10.25	7.506	1.365	16.50	8.27
4	4.82	3.529	1.366	16.46	8.30
5	5.11	3.848	1.328	16.46	8.07
6	2.86	2.094	1.366	16.49	8.28
7	3.00	2.227	1.347	16.44	8.19
8	1.27	0.919	1.382	16.45	8.40
9	1.00	0.746	1.340	16.49	8.12
10	2.53	1.859	1.361	16.56	8.22
Average . . . . .			1.355	16.49	8.22

value would probably be nearer the true value, since impurities and deterioration of hemoglobin tend to lower the capacity per gm. value. Our crystalline preparations were highly purified and the time of preparation was shortened to a maximum of 18 hours as compared to 2 or 3 days by other workers. (3) With non-crystalline and presumably less pure preparations of human and ox hemoglobin we were able to obtain values as high as the generally

<sup>3</sup> Carbon monoxide capacity is identical with oxygen capacity as shown by Sendroy, Dillon, and Van Slyke (11).

accepted capacity of 1.34 cc. per gm. (4) Recent work (8, 9) places the spectrophotometric extinction coefficient at a higher value than used by previous workers, and this correction would increase their capacity values.

Hemoglobin is one of the few proteins whose molecular weights can be determined by chemical methods. It would thus serve as a standard for molecular weight determinations on other proteins by physical methods. The equivalent weight of hemoglobin calculated from our data is 16,470 and the molecular weight is 65,880 compared with Hüfner's values of 16,670 and 66,680. Direct molecular weight determinations are not as precise as most methods of chemical analysis and, with the exception of the Svedberg (28) method, depend on a primary standard; *i.e.*, for hemoglobin, oxygen capacity, iron, or total nitrogen. While direct molecular weight determinations of hemoglobin are necessary in estimating what multiple of the equivalent weight is the true molecular weight, they cannot be used in calculating a precise value for the oxygen capacity of hemoglobin.

We are cognizant of the fact that the values we present are based upon a product obtained when active hemoglobin is dried. The solubility is altered and the product is no longer active towards oxygen or carbon monoxide. These conditions are true of all previous work. We have demonstrated repeatedly that there is no loss of nitrogen on drying; we have not given specific data, as these would merely recapitulate the total nitrogen data. Our data do not settle the question of a possible loss of hydrogen and oxygen in the form of water which may be essential to activity of the molecule. But such loss of water, if it occurs, is constant and does not affect the reproducibility of this practical standard.

#### SUMMARY

1. Direct and indirect measurements of the carbon monoxide capacity of dog hemoglobin indicate a minimal value of 1.36 cc. of CO per gm.
2. The total nitrogen of dog hemoglobin is  $16.49 \pm 0.08$  per cent.
3. The molecular weight of hemoglobin calculated from chemical data is approximately 66,000.
4. Drying hemoglobin at  $105^{\circ}$  does not result in a loss of nitrogen from the molecule.



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# THE SOLUBILITIES OF CERTAIN AMINO ACIDS AND RELATED COMPOUNDS IN WATER, THE DENSITIES OF THEIR SOLUTIONS AT TWENTY-FIVE DEGREES, AND THE CALCULATED HEATS OF SOLUTION AND PARTIAL MOLAL VOLUMES. II\*

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The present work is a continuation of the previously reported studies on the solubilities of various amino acids in water and the densities of their aqueous solutions (1).<sup>1</sup> Data are here presented for *l*-asparagine monohydrate, *l*-cystine, *d*-isoleucine, *dl*-methionine, *l*-phenylalanine, *dl*-serine, taurine, and *l*-tryptophane. Except for asparagine, previously reported data relating to these compounds are either quite fragmentary or inconsistent.

The purity of all compounds was such as to meet the requirements of the present experiments.<sup>2</sup> The technique used in the determination of solubilities and densities, the method of calculating the equations for the solubility data, and the calculation of the differential heats of solution and the partial molal volumes were the same as those which have been described previously (1). The micro-Kjeldahl procedure of Parnas and Wagner (2) was used to estimate the nitrogen content of the tryptophane solutions,

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<sup>1</sup> On p. 554, last line of the text, a typographical error should be corrected by substituting *V* for  $\varphi$ . The nomenclature used throughout the present paper is the same as that previously given (1).

<sup>2</sup> The optical activity,  $[\alpha]_D^{25}$ , of the compounds studied was determined as follows: *l*-asparagine  $\cdot$  H<sub>2</sub>O, +37.3° in 10 per cent HCl; *l*-cystine, -223° in 1 N HCl; *d*-isoleucine, +36.3° in 20 per cent HCl; *l*-phenylalanine, -35.0° in H<sub>2</sub>O; *l*-tryptophane, -33.2° in H<sub>2</sub>O.

while cystine was determined by Okuda's (3) method. In all other cases, the amount of solute was determined by drying a weighed sample of the solution. The solubilities of methionine, serine, and taurine were determined over a temperature range of 0–62°, and the other compounds over the range of 0–65°. The solubility of taurine was determined at ten, tryptophane at eight, and the other compounds at nine different temperatures. The following number of determinations was made: asparagine 26, cystine 20, isoleucine 18, methionine 36, phenylalanine 26, serine 32, taurine 34, tryptophane 18.

The solubility equations are given in Table I. These equations were used to calculate the solubility data, at 5° intervals, which are given in Table II. The density data at 25°, together with the values for the partial molal volumes of the solute,  $\bar{v}_2$ , and, when possible, the values calculated for this quantity from Traube's atomic volumes (4), are given in Table III. The value for the partial molal volume of the solvent  $\bar{v}_1$  was 18.069 in each instance. The coefficients of the equations used to calculate values for  $\Delta H$ , the values for this quantity, and, when possible, the observed calorimetric values are found in Table IV. Since the activity coefficient of none of the compounds studied has been determined, the calculated values are to be regarded as only approximate.

#### DISCUSSION

*l*-Asparagine·H<sub>2</sub>O—Three solubility studies are reported in the literature. Those of Guareschi (5) and of Bressler (6) agree within a few per cent of the present data. The data cited by Cook (7) are, however, irrationally low, being in the neighborhood of 15 per cent of the present values.

*l*-Cystine—The solubility values for *l*-cystine reported in the literature are quite variable. Neuberg and Mayer (8), Sano (9), Mörner (10), Blix (11), and du Vigneaud (12) report values that agree well with the present work.<sup>3</sup> However, values reported by Pfeiffer and Angern (13), Toennies and Lavine (14), Andrews and de Beer (15), and Hoffman and Gortner (16) deviate between 20 and 80 per cent from the present data.

<sup>3</sup> The solubility value for *l*-cystine found by E. J. Cohn is 0.109 gm. per liter of water at 25°. This is essentially the same as found in the present studies. (Personal communication.)

TABLE I  
Coefficients of Solubility Equations\* of Certain Amino Acids and Related Compounds

Substance	$a_1$	$b_1 \times 10^2$	$c_1 \times 10^5$	$a_2$	$a_3$	$b_2 \times 10^2$	$c_2 \times 10^5$	$a_4$	$b_4 \times 10^2$	$c_4 \times 10^5$	Maximum deviation†	Mean deviation†
											per cent	per cent
L-Asparagine-H <sub>2</sub> O.....	0.9289	2.311	-4.981	-1.2475	-25.9584	1.159	-11.47	-30.2463	1.179	-11.84	+2.47	±1.00
L-Cystine.....	-1.299	1.357		-3.680	-18.643	3.125		-21.023	3.125		-2.12	±1.01
D-Isoleucine.....	1.5787	0.07862	2.594	-0.5389	2.7190	-3.081	5.972	-1.3913	-3.020	5.866	-2.72	±1.26
D-Methionine.....	1.2597	1.108	1.221	-0.9140	-11.1682	4.086	-2.811	-15.2099	4.111	-2.871	+2.26	±0.73
L-Phenylalanine.....	1.2974	0.6982		-0.9204	-6.510	1.608		-10.5103	1.601		-2.40	±1.15
D-Serine.....	1.3432	1.520	-3.548	-0.6782	-17.2153	7.963	-8.169	-21.4529	8.134	-8.504	-1.43	±0.56
Taurine.....	1.5945	1.916	-8.500	-0.5029	-27.8015	15.10	-19.57	-32.1283	15.35	-20.07	-1.98	±0.86
L-Tryptophane.....	0.9156	0.4834	2.988	-1.3942	-11.3824	4.872	6.881	-15.3928	4.869	6.879	+5.41	±1.44

\* Solubility equations:  $\log S = a_1 + b_1 t + c_1 t^2$ ;  $\log m = a_2 + b_2 t + c_2 t^2$ ;  $\ln m = a_3 + b_3 T + c_3 T^2$ ;  $\ln N_2 = a_4 + b_4 T + c_4 T^2$ .

† Maximum deviation of the observed from the calculated values.

‡ Calculated from the formula, mean deviation =  $(\Sigma D^2/n)^{1/2}$ .

As is well known (8), *l*-cystine is racemized when heated in the presence of strong acid. It is not surprising then that cystine prepared from hair or wool by hydrolysis with strong HCl is contaminated with inactive isomers. Unless particular care is taken

TABLE II

*Table of Calculated Solubilities of Certain Amino Acids and Related Compounds in Water\**

<i>t</i>	<i>l</i> -Asparagine·H <sub>2</sub> O	<i>l</i> -Cystine	<i>d</i> -Iso-leucine	<i>dl</i> -Meth-ionine	<i>l</i> -Phenyl-alanine	<i>dl</i> -Serine	Taurine	<i>l</i> -Tryptophane
°C.								
0	8.49	0.0502	37.91	18.18	19.83	22.04	39.31	8.23
5	11.05	0.0587	38.30	20.64	21.50	26.20	48.78	8.72
10	14.29	0.0686	38.83	23.40	23.29	31.03	59.92	9.27
15	18.38	0.0802	39.47	26.50	25.24	36.58	72.91	9.88
20	23.51	0.0938	40.25	29.95	27.35	42.95	87.84	10.57
25	29.89	0.1096	41.17	33.81	29.65	50.23	104.8	11.36
30	37.79	0.1281	42.23	38.12	32.13	58.52	123.8	12.23
35	47.50	0.1498	43.45	42.90	34.82	67.87	144.9	13.23
40	59.37	0.1751	44.83	48.24	37.73	78.42	167.8	14.35
45	73.77	0.2048	46.41	54.15	40.89	90.24	192.6	15.62
50	91.18	0.2394	48.18	60.70	44.31	103.4	218.8	17.06
55	112.0	0.2799	50.17	67.95	48.02	118.0	246.2	18.70
60	136.8	0.3272	52.40	75.95	52.04	134.1	274.2	20.57
65	166.2	0.3826	54.88	84.78	56.40	151.8	302.6	22.70
70	200.6	0.4472	57.65	94.52	61.11	171.1	330.5	25.14
75	240.9	0.5229	60.76	105.2	66.24	192.1	357.6	27.95
100	551.7	1.142	82.55	176.0	99.00	322.4	457.6	49.87
$\frac{S_{100}}{S_0}$	65.1	22.8	2.18	9.68	5.00	14.63	11.68	6.07

\* These values were calculated on the basis of the equations given in Table I. The term  $S_{100}/S_0$  is the ratio of the solubility at 100° to the solubility at 0°. All values are in terms of gm. per 1000 gm. of water. The values above 70° are probably less accurate than those below, owing to the fact that no solubility estimations were carried out above 70°.

in the purification of the sample, the solubility values obtained will approximate a summation of the solubilities of *l*-, *dl*-, and mesocystine.

The cystine used in the present work was prepared from a cystine stone, care being taken that the preparation should not be in contact with acid any longer than was absolutely necessary. The

preparation was then twice crystallized from hot water. Of this product, 10 mg. were placed in 60 ml. of boiled distilled water and the solubility was determined. Likewise, a value was obtained

TABLE III

*Density of Aqueous Solutions of Certain Amino Acids and Related Compounds\* at 25° and Partial Molal Volumes of Solute*

<i>d</i> -Isoleucine				<i>d</i> -Methionine			
Composi- tion	Concen- tration	<i>m</i>	<i>d</i>	Composi- tion	Concen- tration	<i>m</i>	<i>d</i>
<i>per cent</i>				<i>per cent</i>			
4.341	45.38	0.3461	1.00579	3.337	34.51	0.2314	1.00678
3.328	34.42	0.2625	1.00374	2.892	29.78	0.1997	1.00547
2.259	23.12	0.1763	1.00152	1.912	19.49	0.1307	1.00258
1.289	13.06	0.0997	0.99953	0.971	9.81	0.0658	0.99987
( $\bar{v}_2 = 105.5$ ; Traube, 107.1)				( $\bar{v}_2 = 106.4$ ; Traube, 106.5)			
<i>d</i> -Serine				<i>L</i> -Asparagine H <sub>2</sub> O			
4.731	49.66	0.4727	1.01708	2.694	27.68	0.1843	1.00677
3.776	39.24	0.3735	1.01299	1.370	13.90	0.0926	1.00193
2.892	29.78	0.2835	1.00915	( $\bar{v}_2 = 97.0$ )			
2.030	20.71	0.1971	1.00552	<i>Taurine</i>			
1.147	11.60	0.1104	1.00184	9.827	1.0898	0.8709	1.04078
( $\bar{v}_2 = 61.6$ ; Traube, 61.1)†				8.467	0.9247	0.7390	1.03418
<i>L</i> -Phenylalanine				7.149	0.7699	0.6153	1.02827
2.877	29.62	0.1794	1.00350	6.121	0.6520	0.5211	1.02366
1.477	14.99	0.0908	1.00035	4.938	0.5195	0.4151	1.01843
( $\bar{v}_2 = 128.7$ )				3.996	0.4162	0.3326	1.01425
				3.010	0.3103	0.2480	1.00995
				2.005	0.2046	0.1635	1.00556
				1.097	0.1108	0.0886	1.00173
				( $\bar{v}_2 = 72.3$ )			

\* The concentration is given as gm. per 1000 gm. of water, *m* is given as moles per 1000 gm. of water, and *d* is the absolute density.

† Cohn, E. J., McMeekin, T. L., Edsall, J. T., and Blanchard, M. H., *J. Am. Chem. Soc.*, **56**, 784 (1934) report 60.8 for the observed apparent molal volume of serine.

with 100 mg. of cystine in the same volume of water. As the results agreed within 3 per cent, the material was considered sufficiently pure for further studies. Solubility is a much more

rigorous test for high degree of purity than the optical rotatory power.

*d-Isoleucine*—The solubility value previously reported by Ehrlich (17) agrees to within 3 per cent of that which was interpolated from the present data.

By comparing the solubilities as summarized in Table II with the corresponding values for *dl*-isoleucine given in the previous paper (1), it will be noted that the optically active isomer is more soluble than the inactive. This, together with the fact that the slope of the solubility curve is less in the case of the active material, should indicate, according to the principles outlined by Meyerhoffer (18), that *dl*-isoleucine is a racemic compound.

TABLE IV

*Calculated and Observed Differential Heats of Solution at 25°, Together with Coefficients Used in the Calculations*

$$\Delta H = RT^2 (a + 10^{-4} bT).$$

Compound	a	b	$\Delta H$ calculated	$\Delta H$ observed*
			calories	calories
<i>l</i> -Asparagine·H <sub>2</sub> O.....	0.11786	-2.3684	8350	8000
<i>l</i> -Cystine.....	0.03125	0	5500	
<i>d</i> -Isoleucine.....	-0.03020	1.173	843	
<i>dl</i> -Methionine.....	0.04111	-0.5741	4240	4100
<i>l</i> -Phenylalanine.....	0.016012	0	2830	
<i>dl</i> -Serine.....	0.08134	-1.701	5410	5050
Taurine.....	0.1534	-4.014	5950	5700
<i>l</i> -Tryptophane.....	0.04869	1.3758	1360	

\* Zittle, C. A., and Schmidt, C. L. A., *J. Biol. Chem.*, **108**, 161 (1935).

*dl*-Methionine—As far as could be ascertained, no previous values on the solubility of *dl*-methionine in water have been reported.

*l*-Phenylalanine—The solubility value for *l*-phenylalanine reported by Fischer and Schoeller (19) at 25° is in good agreement with the present data.

By comparing the solubility values for *l*-phenylalanine in Table II with the corresponding values found previously for *dl*-phenylalanine (1), it will be noted that the optically active isomer is more soluble than the inactive form. According to Meyerhoffer (18), this should indicate that *dl*-phenylalanine is a racemic compound,

although the close similarity of the slopes of the solubility curves may cast some doubt on this conclusion.

*dl-Serine*—The one previously reported value for the solubility of *dl-serine* in water by Fischer and Leuchs (20) agrees very closely with the present data. The material used in the present studies was kindly supplied by Dr. M. S. Dunn.<sup>4</sup>

*Taurine*—Previous solubility data for taurine are limited to a single determination reported by Gmelin (21). The agreement is within 1 per cent of the value interpolated from the present work.

*l-Tryptophane*—With the exception of the single determination given by Pfeiffer and Angern (13), no definite values concerning the solubility of *l-tryptophane* in water are reported in the literature. Interpolation of the present data indicates that this value is approximately 20 per cent high.

The material used in the present work was generously supplied by the Pfanstiehl Chemical Company.<sup>5</sup> It was recrystallized several times from water in an atmosphere of nitrogen to eliminate the darkening that usually takes place as a result of oxidation. For the solubility estimations, freshly boiled distilled water was used as the solvent and the air in the solubility tubes was swept out with a stream of nitrogen. Identical solubility values obtained by using either a small or a large excess of solid phase indicated that the material was pure and that the technique employed was satisfactory.

#### SUMMARY

1. The solubilities of the following compounds have been determined over various given temperature ranges: *l-asparagine*·H<sub>2</sub>O, *l-cystine*, *d-isoleucine*, *dl-methionine*, *l-phenylalanine*, *dl-serine*, taurine, and *l-tryptophane*.

2. Equations have been devised for each compound to express the solubility as a function of temperature. The values calculated from these expressions usually deviate from the observed values less than 1.5 per cent.

3. The heats of solution of the compounds mentioned above were

<sup>4</sup> The writers desire to express to Dr. M. S. Dunn their appreciation for the supply of *dl-serine*.

<sup>5</sup> The writers desire to express to the Pfanstiehl Chemical Company their appreciation for the supply of *l-tryptophane*.



calculated and in some instances were compared with direct calorimetric measurements.

4. The molecular volumes of certain of the compounds given above have been calculated from Traube's empirical atomic volumes. A comparison of the values so obtained with those found by the estimation of the partial molal volumes from the density measurements has been made.

5. On the basis of Meyerhoffer's criteria, it is concluded that *dl*-isoleucine and *dl*-phenylalanine are probably racemic compounds.

6. A table giving the solubilities of the compounds named above at 5° intervals from 0–75° and at 100° has been included (Table II).

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# THE SUBDIVISION OF THE METABOLIC NITROGEN IN THE FECES OF THE RAT, SWINE, AND MAN

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The metabolic nitrogen of the feces includes the nitrogen originating from a variety of sources, such as epithelial cells, bacteria, mucus, and the residues from the bile and digestive juices. A liberal interpretation of the term might even include, under abnormal conditions, substances such as pus and blood. That the metabolic nitrogen actually exists has been amply demonstrated by Rubner ((1) p. 198) and others (2-5) by the use of nitrogen-free diets. Various means have been devised to separate the food residue nitrogen from the metabolic nitrogen in the feces in attempts to estimate the true digestibility of dietary protein. Several chemical methods (6-8) for distinguishing the quantities of nitrogen from these two general sources are based upon assumptions concerning the solubility of part of the fecal nitrogen in various reagents for which no adequate support may be found.

In his method of determining the biological values of proteins, Thomas (3) used three formulas in the computation of biological values, involving three different assumptions concerning the amount of metabolic nitrogen in the feces. In his first formula he considered that none of the nitrogen in the feces was metabolic nitrogen; in the second, he considered all of the nitrogen in the feces to be metabolic nitrogen; and in his third formula he estimated the metabolic nitrogen at a constant average value, determined by feeding a nitrogen-free diet. Martin and Robison (4) took this latter view-point, although they realized that the amount of indigestible matter consumed increased the excretion of metabolic nitrogen. Also, Boas Fixsen and Jackson (9) state, ". . . in our work, the same rat has shown an excretion of faecal

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nitrogen which is roughly constant in amount, in spite of large variations in the food intake." The author (10) has presented evidence to the contrary elsewhere.

The work of F. Voit (11), who was able to show with the dog that there is a nitrogenous deposit in a separated loop of intestine, proportional to fecal nitrogen excretion, implies a verification of only a constant fraction of metabolic fecal nitrogen, although his work may also be interpreted as indicating the possibility of a fraction related to the amount of food consumed. Likewise, the fasting experiments with dogs carried out by Müller (12) and C. Voit (13) lead to the conclusion that there is a constant excretion of metabolic nitrogen, although their data are extremely variable, because under conditions of fasting the excretion of feces is very irregular. Nevertheless, it is highly significant that even with these irregularities a relationship may be distinguished between the quantity of fecal nitrogen during fast and the body weight of the dogs. On the contrary Paton and Stockman (14) and Benedict (15) report controlled human fasts of 30 and 31 days in length in which no feces were excreted. Mendel and Fine (16) and Benedict agree that because of diminished peristalsis, "Fasting feces are in great part derived from retained fecal matter, resulting from food immediately preceding the period of inanition." Certainly these longer fasts are more valid than those of shorter duration in deciding this point, and the conclusions of Benedict and of Mendel and Fine appear to be justified. However, it must be recognized that there may be a species difference in this respect, and that genuine fasting feces may actually occur in dog experiments and not appear in human fasts.

The work of Mitchell (5, 17) shows that the total metabolic nitrogen in the feces of rats is fairly proportional to the food intake. He computes the mg. of nitrogen in the feces excreted per gm. of dry matter consumed with a nitrogen-free diet, and then multiplies this value by the gm. of an experimental diet eaten, in order to estimate the metabolic nitrogen in the feces of the latter. However, he finds (17) a slight relationship between the amount of metabolic nitrogen per unit of weight of nitrogen-free diet and the body weights of rats. He also observed that if the amounts of food consumed are small compared to the energy requirement of the rat, abnormally large values are obtained for the ratio of

metabolic fecal nitrogen to the dry matter consumed, a fact that is indicative of the existence of a constant fraction of the metabolic fecal nitrogen. Thus, Mitchell's results are in agreement with the hypothesis that the metabolic nitrogen, in rats at least, is the resultant of two distinct body functions, and on this basis may be subdivided into two fractions: (a) a true excretory fraction that continues at a constant level, even during fast, and that may be related to the basal nitrogen metabolism of the body, and (b) a digestive fraction that is secreted as a result of the ingestion of food and in amounts proportional to the dry matter consumed.

If the metabolic nitrogen were all proportional to food intake, the relationship might be shown by Chart 1-A in which

$$y = bx \quad (1)$$

$y$  being the metabolic nitrogen,  $x$  the dry matter consumed, and  $b$  the slope constant. However, Chart 1-B illustrates the relationship between the metabolic nitrogen and the food intake on the basis of the hypothesis stated above. The relationship between metabolic nitrogen and food intake is in this case expressed by the equation

$$y = a + bx \quad (2)$$

the constant  $a$  being the  $y$  intercept, representing the constant fraction of the metabolic nitrogen. Line  $ac$  in Chart 1-B represents what might be expected if there were only the constant fraction of the metabolic nitrogen.

The metabolic nitrogen per unit of dry matter consumed ( $1/k$ ) at different levels of food intake, as illustrated by Chart 1-A, could be expressed as follows:

$$1/k = 1'/k' = 1''/k'' \dots \text{etc.}$$

The same relationship with regard to Chart 1-B would be expressed thus:

$$1/k < 1'/k' < 1''/k'' \dots \text{etc.}$$

E. Voit (18) attempted to coordinate food intake, expressed as a percentage of the energy requirements of the subject, and fecal nitrogen, expressed in mg. per sq. m. of body surface, of different individuals, and arrived at a graph similar to Chart 1-B. He

labeled line *ac* the *Hungerwert*. Strangely enough, however, he expresses the relationship between metabolic nitrogen and food intake by an equation like Equation 1, such as is described only in Chart 1-A in which the *y* intercept is 0, although in all of his figures it appears that the *y* intercept is a significant positive value. Some of the data of Müller (12) and Rieder (19) with dogs, which Voit expresses per sq. m. of body surface in order to group several animals together, have enough values per individual to fit a very good linear regression from (and including) the fasting value through the data for the various levels of food intake. These experiments verify in their entirety the postulate of a dual origin of the metabolic fecal nitrogen. Voit, however, expressed the food

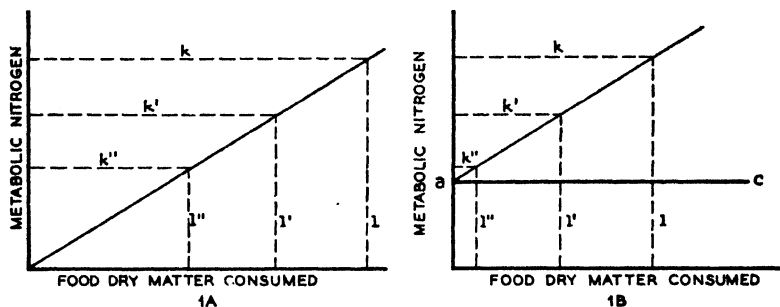


CHART 1. Possible relationships of metabolic nitrogen to food intake

intake as a percentage of the energy requirements of the animals. On this basis he obtained seemingly much less influence of fat than of the protein and carbohydrate on the metabolic fecal nitrogen. If the intakes of these nutrients were expressed in terms of dry matter, as has been done in this laboratory, instead of their calorific value, the effect of fat on the metabolic nitrogen would much more nearly approximate that of the other nutrients. Mitchell (20) has lately obtained definite evidence that fats and carbohydrates in the diet affect the metabolic nitrogen in the feces of rats in proportion to their dry weight, not their caloric content.

The work of Titus (21) with steers is interesting because of the use made of extrapolation. The nitrogen content of the feed was varied (by successive substitutions of nitrogen-free paper pulp for

equivalent amounts of alfalfa hay), a constant intake of food dry matter being maintained, the data being plotted with food nitrogen as abscissas and fecal nitrogen as ordinates. He shows that under these conditions "a linear relationship exists between the nitrogen content of the feces of a steer (when corrected to a uniform water content) and the nitrogen content of its feed . . ." It would follow therefore on the basis of this linear relationship that the  $y$  intercept (from data corrected as stipulated) would represent the metabolic nitrogen for that level of food intake. Because this estimate does not agree with another computed from another formula based on other assumptions, does not discount, much less disqualify, the significance of the  $y$  intercept value. If the linearity holds (as concluded above), the  $y$  intercept which represents a nitrogen-free ration, holds also, and it appears that these data favor rather than disqualify (as Titus concludes) nitrogen-free rations as a means of determining metabolic nitrogen.

It was the purpose of the experiments to be described below to study, with different species of animals, the factors determining the amount of metabolic nitrogen excreted in the feces, and to test the validity of the method of estimating this amount in determinations of the biological value of protein, from the excretion of fecal nitrogen on a nitrogen-free diet, or its equivalent.

*Rat Experiments*—As the constant portion of the metabolic nitrogen has been found to be difficult to measure directly during fasting, because of the irregularities of excretion (12, 13, 19), it was proposed first of all to study the variation in the fecal nitrogen excretion when the intake of a nitrogen-free diet was varied in ten to twenty steps from just above fasting (less than 0.5 gm. of dry matter per day) to maximum consumption (varying with the size and appetite of the rat). If these values are plotted, with the gm. of dry food intake as abscissas and the mg. of metabolic nitrogen as ordinates, the  $y$  intercept of a straight line fitted by the method of least squares would represent the constant portion of the metabolic nitrogen, if such exists. The validity of this indirect method of estimating this fraction of the metabolic nitrogen would depend upon the existence of a rectilinear relationship between total metabolic nitrogen and dry matter intake. Six adult male rats were used in the first series of experiments.

In a second series of experiments, involving forty-five rats rang-

ing in weight from 28 to 444 gm., it was planned to establish the slope and position of the line defining the relation between metabolic fecal nitrogen and intake of dry matter, but with only two determinations per rat. Should the  $y$  intercepts of these lines be significant positive values, and be proportional to body size, total body nitrogen, and endogenous urinary nitrogen, this would constitute evidence that the  $y$  intercept was related to basal nitrogen metabolism.

This plan of procedure required a technique for sharply dividing the feces of one level of feeding from those of the previous and following levels. This necessitated some investigation to determine the best substances which might be used to demarcate the feces of one period from those of another. It was considered desirable to have two feces markers of different colors, but very similar in physical and chemical properties. For the purposes of this study, a good feces marker must (1) distinctly mark the feces resulting from the food with which the marker was fed; (2) be insoluble (hence have less tendency to diffuse through the chyme causing a blending rather than a sharp demarcation, and not be absorbed by the intestines); (3) have no toxic, laxative, costive, or other physiological effect on the subject; (4) not contain or react with the element or elements under investigation (in this case nitrogen, and for this reason bismuth subnitrate, for instance, was not tried).

Lampblack, carmine, barium sulfate, copper sulfate, bismuth subcarbonate, and purple, green, and yellow cellophane, all of which color feces more or less, were tried and were eliminated one by one, because they did not satisfy the above criteria. A search of the literature did not reveal any other materials suitable for marking rat feces. The two substances which were eventually tried and which proved the most successful were chromic oxide and ferric oxide, ( $\text{Fe}_2\text{O}_3$ ), which colored the feces a bright green and red, respectively. These are both used as paint pigments and have marked coloring effects. Further, they are substances of very similar chemical properties, both being almost completely insoluble and neither appearing to have a physiological effect on rats.

The method used was to include either ferric oxide or chromic oxide at a 2 per cent level (or 3 per cent at very low planes of food

intake) in the first feeding of each period. In all subsequent feedings of the period, barium sulfate was substituted for the coloring material. This substance, also of very low solubility, rendered more exact the separation of the red or green feces. To test the equivalence of the two metallic oxides as feces markers, they were used alternately in a series of four 2 day periods on eight rats with the food intake the same throughout; no significant differences could be detected in the metabolic nitrogen excreted, or in the digestibility of the dry matter of the diet, the feces being related to the food intake solely by their color.

The metabolism cages were the glass crystallizing dishes described by Mitchell (5), the rat being suspended 2 inches above the bottom of the dish on  $\frac{1}{2}$  inch mesh wire hung by hooks from the edge of the dish. The top of the dish was covered by an inverted 8 inch glass funnel, ventilation being supplied by a suction pump attached to the stem of the funnel. A small Erlenmeyer flask with a drinking tube fused to the bottom was suspended inside the funnel and served as a container for distilled water for the rat. Food was given in a small porcelain cup having a flange inside to restrict wastage.

There are several factors which may cause irregularities in the amount of fecal nitrogen observed, such as (1) the ingestion of hair, (2) coprophagy, (3) the collection of semen with the feces, and (4) the contamination of feces by urine. Several modifications of the apparatus and procedure were designed in order to eliminate these sources of error.

The ingestion of hair may be incidental to the cleaning process, as the white rat cleans itself thoroughly each day. There is, however, reason to believe, from the quantities of hair observed in the feces, that the amount of hair consumed increases as the amount of food is restricted. In either case, the hair excreted in the feces would tend to raise the  $y$  intercept value  $a$ . The hair was removed by finely grinding and sifting the feces. However, even though the nitrogen of the hair itself was thus eliminated, the passage of hair through the alimentary tract would tend to increase the metabolic nitrogen. Since a part of the metabolic nitrogen seems to be proportional to the dry matter intake, the dry matter of the hair consumed, as well as that of the food, would promote its excretion. In order to check this factor an



attempt was made to use a strain of hairless rats<sup>1</sup> (22); but these, being a mutation from wild rats, were very vicious and uncooperative. Only two survived the experiment. The final method adopted in reducing this factor to a minimum was to clip haired rats of the regular stock colony.

The consumption of feces is commonly supposed to be due to lack of some factor or to low caloric intake. It is logical then to believe that the amount of feces eaten would increase with a deficiency in food intake, and this was found to be true in limited observations on rats receiving variable amounts of food. Although the rat may not benefit in receiving an appreciable amount of nutrients from the feces eaten, certainly a perceptible quantity of metabolic nitrogen would be added when the same dry matter passes through the gastrointestinal tract the second time. Furthermore, since coprophagy increases as the food intake decreases, this fact alone would increase the metabolic nitrogen more at lower levels of feeding, thus giving a false estimate of the constant factor in the metabolic nitrogen. The fact that the rats were kept on  $\frac{1}{2}$  inch mesh wire 2 inches above the floor of the cage did not prevent coprophagy. They were observed to take feces in their fore paws immediately on excretion. To prevent access to the anus and completely eliminate coprophagy, the rats were confined in narrow, arched wire tubes at all times except when they were under observation and when food was before them.

Under the conditions of these experiments masses of mucus-like material were found in the bottom of the metabolism dishes almost daily. It was evident from the position of this material below the body of the rat that it did not come from the nostrils or mouth. It was a question then whether to allot it to the feces or urine. At times the dry substance resembled feces in both form and color, but could be distinguished on close examination by its slightly translucent appearance. This discharge was not observed with rats less than 8 weeks of age or under 100 gm. in weight. In 5 day periods with ten male rats weighing from 118 to 300 gm., averages for the different animals ranged from 4 to 27 mg. of the dried substance per rat daily. The quantity of nitrogen averaged

<sup>1</sup> The author desires to express his indebtedness to Dr. E. Roberts, Genetics Laboratory, University of Illinois, for furnishing these hairless rats.

from less than 1 to more than 4 mg. per day. The average nitrogen content of the dried material was  $16.44 \pm 0.76$  per cent. If this is expressed as conventional protein ( $N \times 6.25$ ), it appears that the substance in question is pure protein.

To seek a further solution to the problem, rats were then rigidly confined over a washboard-like platform with deep cross fissures placed so that the fissures were at right angles to the body of the rat. Thus, the urine and feces fell into separate compartments. The mucus-like substance was found always in the fissure containing the urine. It was clear that this material came from the penis, and it was strongly suspected of being a seminal discharge. As no female rats were near by, it was difficult to believe that this could be the vaginal plug left by the male in copulation. Nevertheless, after a vigil of many hours a rat was discovered in the act of ejaculation. A microscopic examination revealed spermatozoa. Thereafter, great care was taken that dried, discolored semen, which appeared like feces, was not included with the fecal collections.

If feces are left below the rat for several hours, contamination by urine may be an important uncontrolled factor, especially where one is dealing with small amounts of fecal nitrogen as in many of the periods of this experiment. It might be reasoned that the probability of urine contamination is a constant factor, but as the food intake is decreased the proportion of urine to feces is increased. Further, the amount of nitrogen in the urine increases at levels of food (N-free) intake below the energy requirements of the animal. Thus, if the feces are left exposed to contamination with urine, the metabolic nitrogen may appear higher on low food consumption than can be attributed to physiological causes. Therefore, when the rats were confined in wire tubes to prevent coprophagy, they were supported above (and parallel to) a length of glass tubing 55 mm. in diameter and 185 mm. long. This tubing rested in a shallow tin dish of the same length but of slightly less width, and involved the same principle as that used by Ackroyd and Hopkins (23), except that the apparatus was cylindrical instead of spherical. The feces were deflected to one side by the tubing, while the urine was caught by the tin dish. Little if any feces were excreted during the few hours of the day while food was before the rat and it had freedom of movement.

It was judged that the probability of contamination of feces by urine during that time was extremely low.

Because of the extreme (often unphysiological) quantities of food ingested, particularly on the low levels of feeding, it was thought desirable to have as short experimental periods as possible consistent with accurate determinations of the metabolic nitrogen excreted. One and two feedings per day in 1, 2, and 3 day periods were studied, but for the first series of experiments a plan involving one feeding a day in 2 day periods was finally chosen. Accuracy was possible in such short periods because of the sharp demarcation of the feces by the markers used. In the second series of experiments, in which only two periods were used to determine the food intake-metabolic nitrogen relationship, the periods were 5 days in length, except with the younger rats, with which it was necessary on the low plane of intake to shorten the periods to 3 days. A 3 day intermission was allowed between the low and the high intake periods. The urine collections to determine the endogenous nitrogen excretion were all of 7 days duration, extending 2 days beyond the periods during which the feces were collected. The wire tubes to prevent coprophagy were not found to be necessary during the period when the rat received sufficient food. The feces during these periods were washed with hot, acidified, distilled water to remove any contamination by urine, and the washings were included with the urine. All samples of feces were preserved with sulfuric acid except in the experiments with the first six rats in which the feces were dried to determine the fecal dry matter. The feces of an entire period were analyzed for nitrogen by a modification of the Kjeldahl method. Duplicate or triplicate samples were run in all cases except when the quantity of feces was too small. Weekly urine collections were made up to uniform volume, and aliquot samples taken for analysis.

The diets used in these experiments are shown in Table I. The dry matter of Diet 5 is approximately 60 per cent digestible, hence 40 per cent appears in the feces. Such a diet was found to be most advantageous for very low levels of feeding, as with the first six rats studied, since it permitted the excretion of considerable amounts of feces even when the food intake was considerably less than 1 gm. of dry matter (Chart 2) per day. Diet 20 was used in the second series of experiments wherein the rats were fed

at only two planes of food intake. It will be noticed that there is included in the diets 5.02 per cent dried extracted egg, which is sufficient to furnish 4 per cent protein in the diet. It has been found in this laboratory (24) that egg protein is almost 100 per cent digestible when included in a diet at low levels. As it aids greatly in maintaining the animal during the experiment, it has been included in diets which are otherwise essentially nitrogen-free. The Cellu Flour in the diets is a commercial product that contains about 38 per cent of crude fiber, manufactured by a Chicago firm.

TABLE I  
*Composition of Diets Used in Rat Experiments*

	Diet 5	Diet 20
	<i>per cent</i>	<i>per cent</i>
Dried egg .....	5.02	5.02
Osborne and Mendel salts* .....	4.00	4.00
NaCl .....	1.00	1.00
Sucrose .....	10.00	10.00
Cellu Flour .....	34.00	18.00
Starch .....	18.98	39.98
BaSO <sub>4</sub> , Cr <sub>2</sub> O <sub>3</sub> , or Fe <sub>2</sub> O <sub>3</sub> .....	2.00 (or 3.00†)	2.00
Butter fat .....	10.00	10.00
Lard .....	13.00	8.00
Cod liver oil .....	2.00	2.00

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 557 (1919).

† 3 per cent coloring substance was included in the diet in a few cases as noted in the text.

*Discussion of Rat Experiments*—The lowest average daily food intake for which the metabolic nitrogen was measured for any one rat was 0.28 gm. of dry matter. In the first series of experiments the highest food intake observed was 11.55 gm. of dry matter per day, but the second series of experiments included much larger individuals, and maximum food intakes of over 20 gm. were obtained.

The linearity of the data obtained in the first series of experiments is clearly illustrated by Chart 2. The correlation coefficients of  $0.984 \pm 0.0064$ ,  $0.971 \pm 0.0088$ ,  $0.993 \pm 0.0029$ ,  $0.964 \pm 0.011$ ,  $0.937 \pm 0.019$ , and  $0.924 \pm 0.023$  for metabolic nitrogen and dry

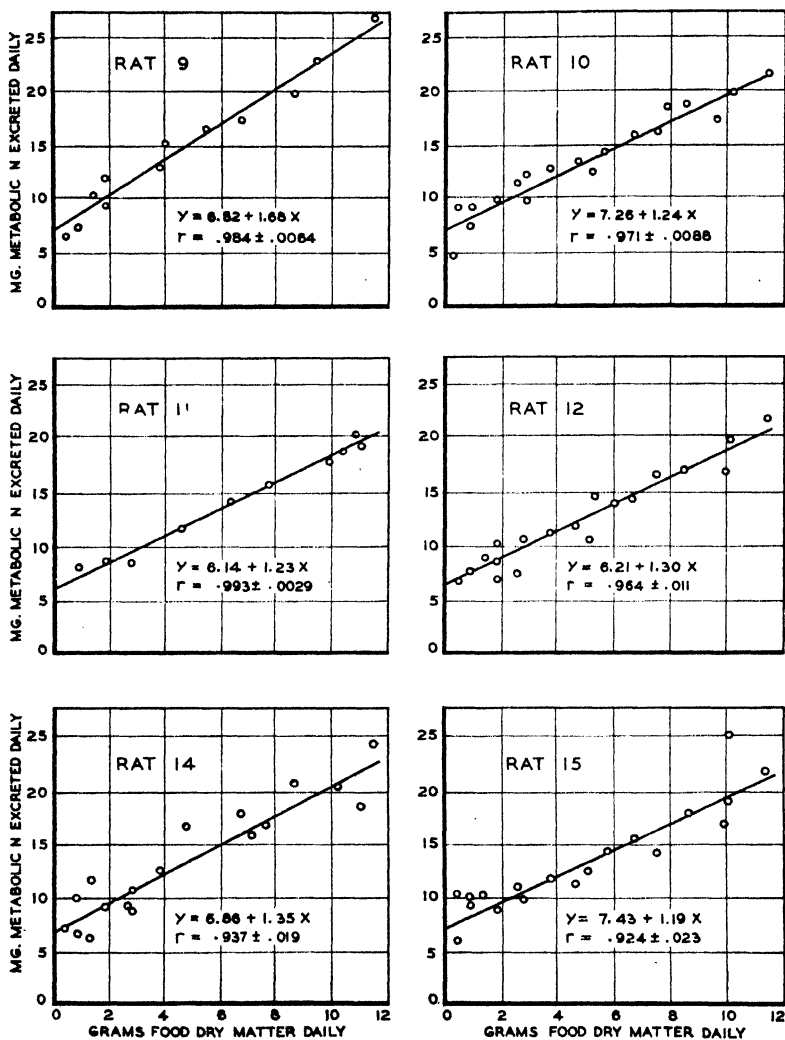


CHART 2. The relationship of metabolic nitrogen to food intake in rats.  $r$  indicates the correlation coefficients.

matter intake for the six rats together with the lack of any consistent trend other than linear in the data indicate that the relation is linear. While it is mathematically possible for the line fitted to these data to depart from linearity beyond the limits which have been investigated experimentally, such departure from linearity is extremely improbable within the limited distance between the lowest plane of food intake and complete fasting. Pendleton and West (25) in their work on the passage of urea between the blood and the lumen of the small intestine believe that the mucosa may act as a simple semipermeable membrane. It might be deduced from their conclusions that as the nitrogen reserves of the body are decreased the amount of urea (and perhaps other nitrogenous substances) passing into the intestinal tract would diminish and hence decrease the metabolic nitrogen. On the contrary, if the metabolic nitrogen of the feces were like the endogenous nitrogen of the urine in this respect, an increase in excretion might be noted when the energy requirements of the animal were no longer met. It would be expected, however, that this change would be initiated at or near the point of calorific maintenance; but the line continues perfectly straight through this point, and there is no reason to believe that it would waver at a point less than 0.5 gm. of dry matter consumed. It seems clear, therefore, that the metabolic nitrogen may be considered a linear function of the dry matter intake.

The apparent lack of connection between energy metabolism and the fraction of the metabolic nitrogen proportional to food intake serves further to disqualify the method employed by Voit (18) of relating metabolic nitrogen to the energy intake expressed as a percentage of the energy requirements of the animal. The fact that no change in the slope of the regression line appears at maintenance, identifies the variable fraction of the metabolic nitrogen more closely with the digestive processes and hence with food dry matter rather than with food energy. Mitchell's (20) recent more direct study of this question has led him to the same conclusion.

It is also clear from Chart 2 that with rats the line describing the relationship between dry matter consumed and metabolic fecal nitrogen excreted has a positive  $y$  intercept, indicating that during a fast fecal nitrogen is still excreted, and that presumably the

fasting excretion continues at a constant rate during periods of feeding. Admittedly the latter indication is merely a reasonable presumption, analogous for example to the presumption that the basal energy metabolism continues unchanged in magnitude during periods of muscular activity or of the ingestion of food.

Having established the existence of two fractions of the metabolic nitrogen, one varying directly with the food intake and the

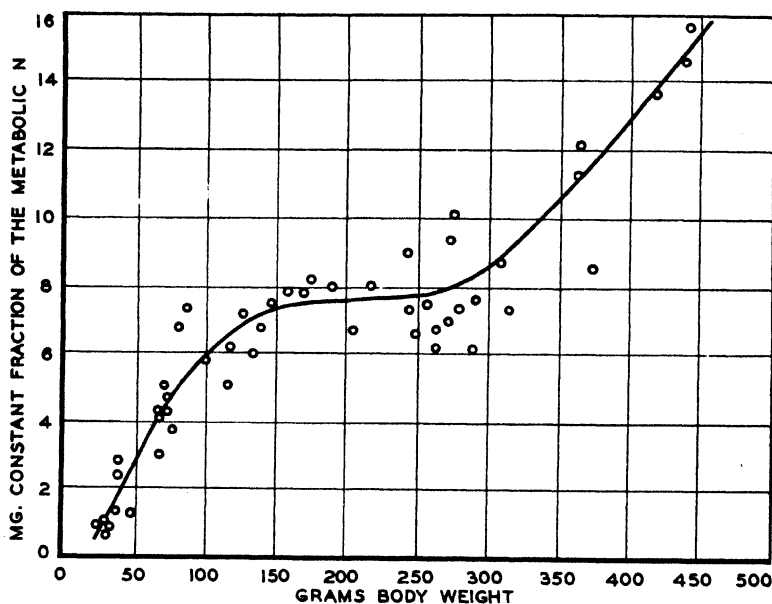


CHART 3. The relationship of the constant fraction of the metabolic nitrogen per day to body weight in rats.

other a constant, it was considered important to determine whether the latter fraction were related to body size or to the magnitude of the endogenous nitrogen metabolism. The second series of experiments was therefore undertaken. Male rats ranging from 25 gm. to 444 gm. in weight were used for this purpose. From the data secured, consisting of two determinations for each rat, the  $y$  intercept values were computed by the method of least squares.

Charts 3 and 4 illustrate the relation between the constant fraction of the metabolic nitrogen (the  $y$  intercept or value  $a$ ) and body weight<sup>2</sup>, and between the same fraction and endogenous nitrogen respectively. The level part of the curve in Chart 3 (and also in Chart 4) relates to rats of 200 to 300 gm. in weight, which is the upper limit of body weight without excessive fatness for rats from

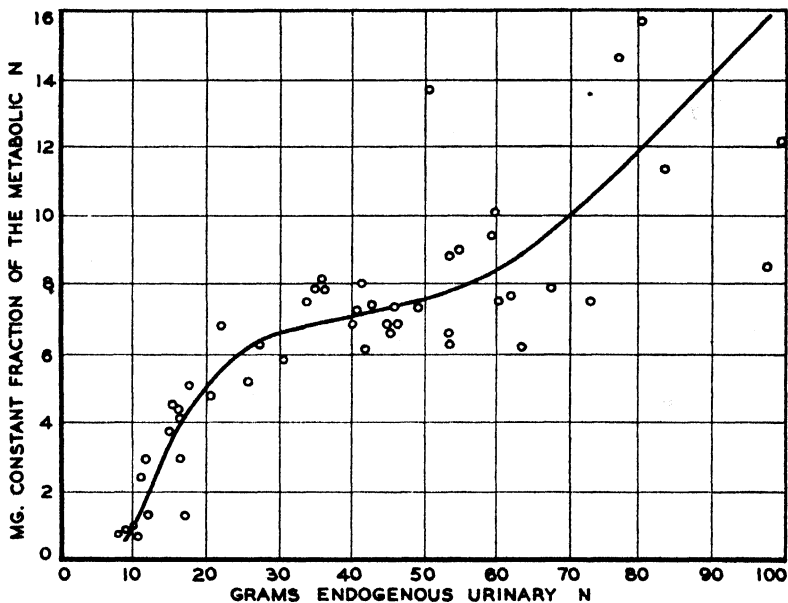


CHART 4. The relationship of the constant fraction of the metabolic nitrogen to the endogenous nitrogen of the urine per day in rats.

the colony at this laboratory. With two exceptions, those animals weighing in excess of 300 gm. came from other laboratories.<sup>2</sup>

The few widely divergent values in Charts 3 and 4, caused by irregularities in the  $y$  intercept values, should not destroy the sig-

<sup>2</sup> The author is indebted to Dr. Julia Outhouse, Department of Home Economics, University of Illinois, and Dr. A. H. Smith and Mr. W. E. Anderson, Laboratory of Physiological Chemistry, Yale University, for kindly supplying these larger animals.



nificance of the greater part of the data. One value was so high that it was not included in the charts. Such infrequent aberrant cases might be caused by blood or pus in the feces, and the author is inclined to believe that such a pathological condition must have existed with three or four of the rats having the most extreme values. The lowest divergent values occurred with those rats which had been longest continued on nitrogen-low diets. Such a phenomenon is observed with endogenous nitrogen determinations when nitrogen-free (or nitrogen-low) feeding is extended for long periods of time (26).

Since there is a close relation between the constant fraction of the metabolic fecal nitrogen and the endogenous nitrogen of the urine, it is suggested that this fraction of the fecal nitrogen be called the "endogenous nitrogen of the feces." As the endogenous nitrogen of the urine is an index of basal nitrogen metabolism, this constant fraction of the fecal nitrogen may also be related to the same phenomena, although not in the same way, since it is not affected by an inadequate energy intake.

The ratio of the constant fraction of the metabolic fecal nitrogen to the endogenous nitrogen of the urine varied from  $1/16$  to  $1/3$ , the average being about  $1/6$ . No simple correlation could be noted between this ratio and the size of the rat, although the data appeared to follow a fairly well defined pattern. The metabolic nitrogen fraction was relatively the smallest ( $1/12$ ) with the youngest rats (less than 40 gm.), rising rapidly to about  $1/4$  with rats weighing 75 gm., then decreasing slowly to  $1/8$  or  $1/9$  with rats weighing from 250 to 300 gm., and increasing with rats over 400 gm., thus describing an S-curve as a function of body weight. No explanation for these variations is offered, and no assurance in their significance, except for the lightest animals, can be felt.

The chart showing the constant fraction of the metabolic fecal nitrogen as a function of the total body nitrogen has not been included in this report, as it is an almost exact duplication of Chart 3. This is not surprising, however, as a correlation coefficient of  $0.993 \pm 0.00087$  between body nitrogen and body weight was computed from nitrogen determinations on 129 rats weighing from 27 to 441 gm., taken from unpublished and published (27) data from this and one other laboratory. Another chart not included is one showing the relationship between the constant fraction of

the metabolic nitrogen and the surface area of the rats. The body surface was calculated by the method of Lee (28) with his body weight-surface area formula,  $S = 12.54W^{0.60}$ . The curve resulting was very similar to that in Chart 3, except for a slight flattening.

Since the metabolic fecal nitrogen appears to consist only in part of constituents varying in magnitude with the dry matter intake,

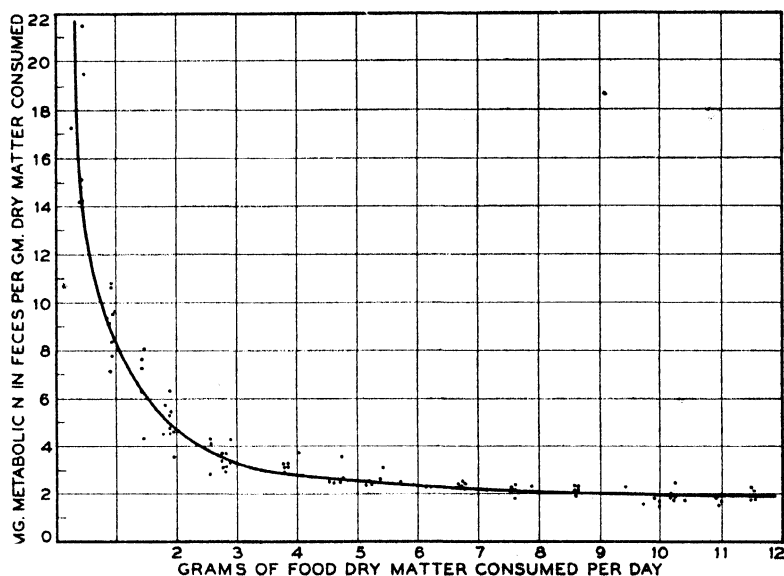


CHART 5. The variation of the metabolic fecal nitrogen per gm. of dry matter consumed with the daily intake of dry matter, for six male rats ranging in weight from 250 to 300 gm.

one might expect the ratio of metabolic nitrogen to dry matter intake to vary with the latter and in an inverse manner. A study of this relationship is important, since the ratio of metabolic fecal nitrogen to dry matter consumed in a period of low nitrogen feeding is used in estimating the metabolic fecal nitrogen in the protein feeding periods of a determination of the biological value of proteins by the Mitchell method (5). In Chart 5 this relationship is shown graphically for the six rats of the first series of ex-

periments. At low levels of food intake, the constant fraction of the metabolic fecal nitrogen predominates, and leads to very high values of the ratio, but when the food intake reaches an approximate maintenance level, the ratio is seen to assume a fairly constant value. Hence, for the purposes of the biological value method, the ratio may be considered constant. The author elsewhere (10) has submitted other evidence to the same effect.

*Swine Experiments*—The selection of subjects for these initial investigations concerned with the origin of the metabolic nitrogen of the feces was restricted to those species (1) that can subsist on purified or nitrogen-free foods, (2) that can eat concentrated foods without roughage since roughage itself is one factor contributing to the metabolic nitrogen, and (3) that have a simple digestive tract (not a ruminant), permitting the sharp demarcation of feces. Swine measure up admirably to these requirements and were accordingly used in the third series of experiments. Twelve pigs of four different breeds, all barrows but one, a Poland China boar, were selected for this purpose. Their range in weight was from 24 to 117 kilcs. The experimental technique was similar to that used in the studies with rats, the food intake of a low nitrogen ration being varied from period to period, and the excretion of fecal nitrogen being measured.

The metabolism crates were of the type designed by Forbes (29). The length of the periods was 3 days generally and occasionally 4 days. The ration consisted of ground cellophane 18 per cent, mineral mixture 4 per cent, yeast 3 per cent, sand, chromic oxide, or ferric oxide 3 per cent, starch 62 per cent, and sugar 10 per cent. To this mixture was added 1 per cent of cod liver oil, which at first was added to the ration at feeding time, but later was incorporated in the ration at the time of its preparation. The mineral mixture consisted of steamed bone-meal 30 per cent, ground limestone 30 per cent, sodium chloride 30 per cent, magnesium carbonate 3 per cent, potassium carbonate 3 per cent, potassium sulfate 2 per cent, ferric chloride 1.5 per cent, and potassium iodide 0.5 per cent. The feces were collected daily, dried at a low temperature, weighed, ground, and analyzed for nitrogen.

10 to 14 days were required for the pigs to become adjusted to the ration. Ferric oxide and chromic oxide were used alternately

as feces markers in the first feeding of the various experimental periods, while sand was used in equal amount in all other feedings. There were no intervening adjustment periods. Although the feces of the pigs were examined for parasites previous to the experiment and negative diagnosis reported, so many round worms were excreted by Pigs 10, 11, and 12 that it was necessary to discontinue the experiment for treatment after the first period. The second period with these pigs was resumed 4 days later.

The results of these investigations are shown in Table II and in Chart 6. It was anticipated that more consistent results might be realized with pigs than with rats, for pigs maintain their weight better on a much more nearly nitrogen-free diet, and eat larger amounts of such food relative to their energy requirements with apparently greater appetite. Proportionately greater extremes in food consumption may be obtained. Also, swine, if healthy and not overfed, eat all of the food put before them immediately, while the feeding of rats usually necessitates keeping food before them for longer periods. For the most successful demarcation of feces the more promptly food is eaten and the longer the time between meals of different periods the better, since less mixing of food of different colors in the digestive tract will be possible. However, the diet, although designed to promote regular excretion, seemed to induce constipation with a few pigs, while with others diarrhea resulted at high levels of consumption. The feces were very high in nitrogen when diarrhea occurred, and for this reason with each of four pigs (Nos. 2, 3, 5, and 9) the period of greatest food intake was not considered in fitting the regression line. Pig 1 was constipated throughout the experiment, and excreted all of the feces of the last two periods during one night. As there was confusion regarding the division of the feces of the two periods under these circumstances, these two 3 day periods were combined as one 6 day period.

The plotted data in Chart 6 indicate, as with rats, that metabolic fecal nitrogen is a rectilinear function of food intake. Furthermore, the regression line for each of the twelve pigs has a positive  $y$  intercept value. Thus, it appears that there is a constant fraction of the metabolic fecal nitrogen with pigs, again similar to the situation with rats. However, with pigs no relationship between body weight and the constant fraction of the

TABLE II

*Summary of Swine Data on Daily Basis*

Pig No., breed, and sex	Body weight	Length of collection periods	Feed dry matter by periods	Fecal N by periods	Constant fraction of metabolic N	Metabolic N per gm. food dry matter
	kg.	days	gm.	gm.	gm.	mg.
1. Poland China barrow	117	4	200	0.30	0.12	1.50
		3	400	0.34		0.85
		3	600	0.56		0.93
		6	1100	0.90		0.82
2. Poland China barrow	54	4	200	0.32	0.23	1.60
		3	400	0.50		1.25
		3	600	0.60		1.00
		3	900	0.80		0.89
		3	1068	0.84		0.79
		3	1288	1.66*		
3. Hampshire barrow	40	4	200	0.37	0.26	1.85
		3	400	0.37		0.92
		3	600	0.51		0.85
		3	800	0.58		0.72
		3	1100	1.24*		
4. Hampshire barrow	49	4	200	0.21	0.11	1.05
		3	400	0.41		1.02
		3	600	0.51		0.85
		3	800	0.65		0.81
		3	1100	0.83		0.75
5. Hampshire barrow	37	4	200	0.23	0.16	1.15
		3	400	0.31		0.78
		3	600	0.41		0.68
		3	800	0.45		0.56
		3	1100	1.38*		
6. Hampshire barrow	34	3	200	0.35	0.16	1.75
		3	400	0.31		0.78
		3	600	0.46		0.77
		3	800	0.57		0.71
		3	1100	0.81		0.74
7. Poland China boar	115	3	319	0.43	0.26	1.35
		3	683	0.71		1.04
		3	1093	1.01		0.92
		3	1502	1.22		0.81
		3	2003	1.53		0.76

\* These values are not included in Chart 6 or used in computing the regression line, as abnormally large amounts of nitrogen were excreted because of diarrhea.

TABLE II—*Concluded*

Pig No., breed, and sex	Body weight	Length of collection periods	Feed dry matter by periods	Fecal N by periods	Constant fraction of metabolic N	Metabolic N per gm. food dry matter
	kg.	days	gm.	gm.	gm.	mg.
8. Duroc Jersey barrow	59	3	182	0.68	0.46	3.74
		3	455	0.94		2.06
		3	820	1.24		1.51
		3	1275	1.57		1.23
		3	1680	2.25		1.34
9. Chester white barrow	68	3	228	0.40	0.20	1.75
		3	455	0.24		0.53
		3	820	0.76		0.93
		3	1275	0.81		0.64
		3	1685	1.80*		
10. Hampshire barrow	34	3	182	0.46	0.47	2.53
		2	364	0.78		2.14
		3	728	0.76		1.04
		3	1184	1.25		1.06
11. Duroc Jersey barrow	35	3	182	0.31	0.13	1.70
		2	364	0.46		1.26
		3	723	0.59		0.82
		3	1184	1.16		0.98
12. Chester white barrow	24	3	182	0.45	0.29	2.47
		2	364	0.54		1.48
		3	692	0.83		1.20
		3	510	0.73		1.43

metabolic fecal nitrogen can be detected from these data, though with rats a somewhat close relationship existed. It was perhaps unfortunate that a greater number of larger pigs were not available for this study; possibly a relationship might have been revealed if such had been the case.

The last column of Table II contains the ratios of the metabolic nitrogen in the feces to the dry matter consumed. This ratio for pigs shows the same general trend as for rats (see Chart 5), large values being obtained for small intakes, but fairly constant values for intakes of food somewhat above what would be required for energy equilibrium.

*Experiments with Human Subjects*—Since the rat and pig experiments were not concordant in all respects, it was deemed important to extend the experiments to another species. Accord-

ingly, five male human subjects of widely different body weights were chosen for investigation. Data regarding the personnel

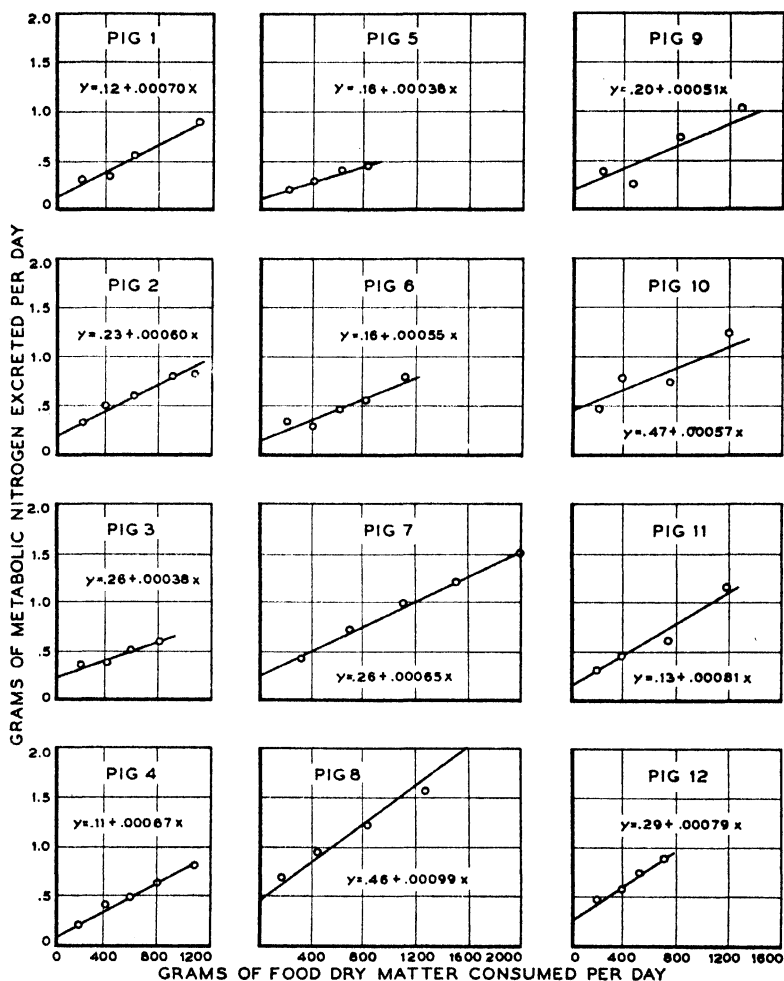


CHART 6. The relationship of metabolic nitrogen to food intake in swine

are given in Table III. All cooperated very well, and the experiment was carried out without mishap. Indeed, it is of scientific

importance that the attitudes of the subjects were good at all times. The inadequate calorific intake of the last period was rendered more apparent because of the extreme cold prevailing at this time, the minimum temperatures ranging from  $-19.4^{\circ}$  to  $-14.0^{\circ}$  with mean daily temperatures of  $-17.7^{\circ}$  to  $-7.2^{\circ}$  (United States Weather Bureau Station, Urbana). One of the group (C.D.M.) fortunately possessed an unusual sense of humor which constantly overcame possible slight tendencies of anyone to grumble about the restricted diet. The meals were always times

TABLE III  
*Summary of Data on Human Subjects on Daily Basis*

Subject	Age	Weight at end of successive periods	Height	Food dry matter by periods	Fecal N by periods	$y$ intercept values in terms of N	Fecal N per gm. food dry matter
	<i>yrs.</i>	<i>kg.</i>	<i>m.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>
J.D.M.	17	53.3	1.56	656	5.49	+0.40	8.37
		52.1		431	3.84		8.90
		50.8		212	2.00		9.44
C.D.M.	29	90.9	1.86	861	4.63	+0.43	5.37
		89.0		566	3.45		6.09
		87.2		279	1.74		6.23
H.R.M.	21	69.0	1.82	780	5.72	-0.11	7.34
		67.8		512	3.81		7.44
		66.6		252	1.75		6.94
L.L.S.	19	58.8	1.79	748	4.84	-0.66	6.47
		58.1		492	2.46		5.00
		56.3		243	1.24		5.11
B.H.S.	31	70.4	1.80	738	5.45	+0.02	7.38
		69.7		485	3.68		7.58
		68.2		239	1.75		7.31

of congenial comradeship, and frequently of hilarity. Outside of meal hours, the subjects attended classes and carried out their usual duties. When on the campus the subjects used a vacant room of the laboratory as a lavatory, and collections at other times were delivered promptly to the laboratory.

No attempt was made to make the diet nitrogen-free. The aim in the experiment was to have a uniform and low intake of nitrogen in a very digestible form. There was very little nitrogen of vegetable source. The principal sources of protein were purposely



introduced in eggs and milk, which proteins are almost 100 per cent digestible (24). The subjects were permitted to drink water from the city water supply, which contained 4.68 parts of nitrogen per million, an amount of nitrogen too insignificant to affect the data desired. With only one subject (C.D.M.) did the total daily nitrogen intake exceed 6 gm. (6.1 gm.), and this was in the first period when the quantity of food consumed was the greatest. The meals<sup>3</sup> were the same for each day as follows:

Breakfast	Lunch	Dinner
Cod liver oil	Lettuce and tomatoes	Eggs
Orange juice	Bananas and cream	Sweet potatoes
Biscuits	Milk	Mousse
Butter	Biscuits	Tea
Jam	Butter	Biscuits
		Butter

The biscuits were planned to be nitrogen-free and were made from a batter containing starch 50 parts, shredded agar 10 parts, Cellu Flour 10 parts, sugar 6 parts, salt 2.6 parts, lard 12 parts, baking powder 2.6 parts, and water 88 parts. The biscuits were weighed and sampled each meal before baking, and all other foods were sampled as they were weighed or measured for the table. The eggs were scrambled with cream, and the sweet potatoes mashed, a small amount of water being incorporated as necessary before each meal in order to keep the moisture content always approximately the same.

The experiment lasted 11 days. There was one preliminary day, then three 3 day periods, and a final day. The University Health Service advised against the use of chromic oxide and ferric oxide for human subjects, so carmine was fed as a feces marker. From 0.2 to 0.6 gm. of carmine was given in capsules to each of the subjects at breakfast on the 2nd, 5th, 8th, and 11th mornings.

<sup>3</sup> The author wishes to express his indebtedness to Dr. Julia Outhouse, Mrs. C. R. Meyer, and Miss Janice M. Smith, Department of Home Economics, University of Illinois, who were responsible for the preparation and serving of the meals. Every effort possible was made to have the table and food attractive, and to take away any thought of the restrictions of the experiment, as well as to preserve the accuracy of the technique,

During the first period the subjects were encouraged to gorge themselves, emphasis being placed upon the nitrogen-free foods, the only restrictions relating to the quantities of milk and eggs to be eaten. All ate to capacity. The approximate daily caloric intakes during this period were: J.D.M. 4050; C.D.M. 5260; H.R.M. 4740; L.L.S. 4630; B.H.S. 4450. In the second period, each person was limited each day to two-thirds the average daily quantity (on the fresh weight basis) of each food eaten during the first period. In the third period, the food intake was limited to one-third that of the first period, the proportions of the various foods continuing the same throughout.

The total dry matter ingested and the nitrogen of the feces excreted by each of the five subjects per day in the three periods is shown in Table III. The relationship between the two is shown graphically in Chart 7. The linearity of this relationship is clearly indicated, even more clearly than was the case with the rat data or the pig data.

The fecal nitrogen  $y$  intercept values are both positive and negative and do not indicate that the hypothesis of a dual origin of the metabolic nitrogen so clearly borne out in the rat and pig experiments is applicable to human subjects. It is not believed, of course, that there is actually a negative  $y$  intercept. Likewise, it does not seem probable that the excretion of the metabolic nitrogen would not start until the fecal excretion has reached a certain level; *i.e.*, that there is only an intercept on the  $x$  axis. The negative  $y$  intercepts can be explained better as being the result of experimental variation, particularly since the method of determination is indirect. Although there were only five human subjects in this experiment, the results so consistently show the  $y$  intercept to be at or near the 0 point, that, with the support of certain fasting experiments (14, 15) already cited, there is established considerable probability that in human feces there is no constant fraction of the metabolic nitrogen, but that all of the metabolic nitrogen is proportional to food intake. Hence, the ratio of fecal nitrogen to dry matter consumed, given in the last column of Table III, is fairly constant and in particular does not show the higher values for low intakes of food exhibited by the rat data and the pig data.

Mitchell ((17) Table I) has cited the results of twenty-two ex-

periments on human subjects involving the feeding of low nitrogen diets, in which the ratio of fecal nitrogen to dry matter consumed ranged from 0.6 to 5.5 mg. per gm., averaging 2.3 mg. If this is

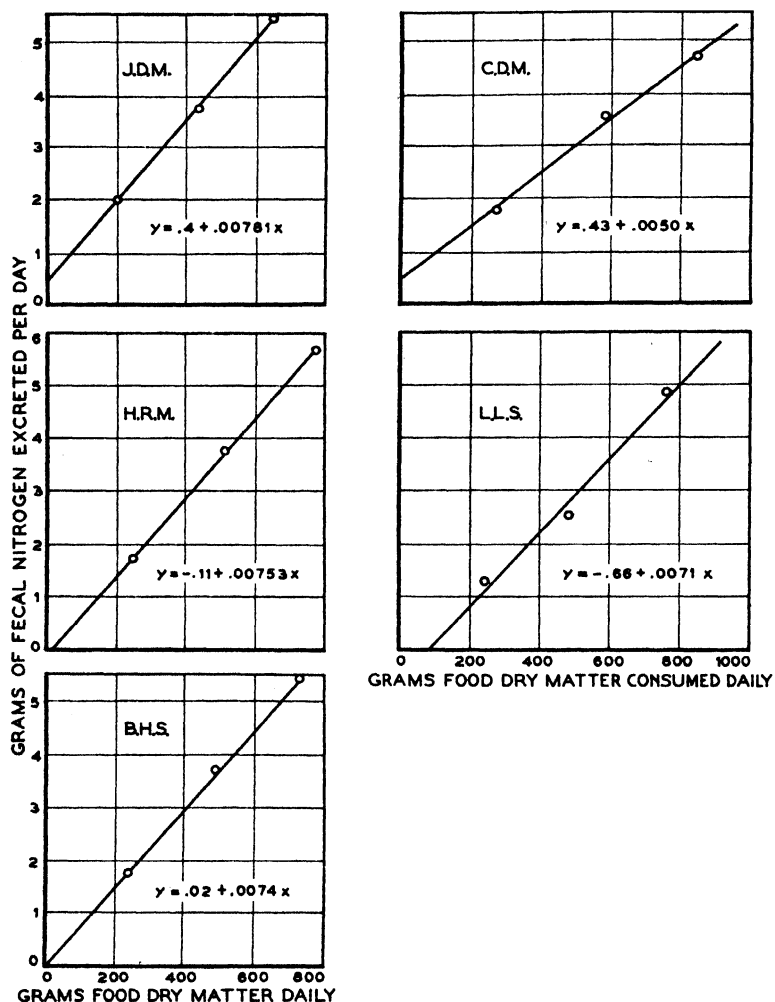


CHART 7. The relationship of metabolic nitrogen to food intake in human subjects.

the order of magnitude of the ratio under conditions such that the fecal nitrogen is all, or nearly all, of metabolic origin, then the uniformly high ratios obtained in this experiment may have resulted from undigested dietary nitrogen, contrary to the expectations based upon the composition of the diet, or they may have been caused by the agar and Cellu Flour in the biscuits together with the large amount of undigestible fibrous matter in the vegetables not usually included in low nitrogen diets. Either case, however, will not detract from the significance above given to the rectilinearity of the relationship of fecal nitrogen to dry matter intake, or to the fact that the  $y$  intercept of the line describing this relationship is 0 within the limits of experimental error.

#### DISCUSSION

In the determination of the biological values of proteins, the ratio of the metabolic fecal nitrogen to the dry matter consumed, determined in a standardizing period, is used in computing the metabolic nitrogen in the feces of experimental periods in which the proteins under test are fed. Nutrition workers (5, 9, 10) have observed this ratio to vary considerably with rats as well as with pigs. It might be argued from data reported in this paper that the metabolic nitrogen of protein feeding periods should be computed by means of an equation such as Equation 2. However, the data from the rat and pig experiments indicate that the usual ratio of metabolic nitrogen to dry matter consumed is fairly constant (Chart 5) if sufficient food is ingested to meet the energy requirements of the animal (10). As this condition is necessary in measuring accurately the endogenous nitrogen of the urine of either rats or pigs, which is usually done simultaneously in the biological value method (5), no modification is suggested for the estimation of the metabolic fecal nitrogen.

The significance of the constant fraction of the metabolic fecal nitrogen is a matter of considerable interest. This fraction was found definitely to vary with body size in the case of rats; in the case of pigs, no such relationship was established, but the conditions for establishing a relationship were much less favorable, there being fewer pigs and less effective variation in size. Expressed as mg. of nitrogen per kilo of body weight, the constant

fraction averaged 41.3 for rats and 5.5 for pigs, but when expressed as mg. of nitrogen per sq. m. of body surface,<sup>4</sup> the averages for the two species were very nearly the same; *i.e.*, 239 and 222, respectively. The latter ratio was the less variable of the two for both rats and pigs, especially, in the former case, if animals weighing less than 50 gm. are excluded. With this exclusion the coefficient of variation of the ratio involving body weight is 37.51, while that of the ratio involving body surface is only 24.47. It may be concluded that the constant fraction of the metabolic fecal nitrogen, like the endogenous urinary nitrogen (26), varies more closely in proportion to body surface than in proportion to body weight, particularly among different species.

Contrary to results on rats and pigs, the results on human subjects indicate that all of the metabolic nitrogen of human feces is proportional to food intake. Furthermore, the ratio of fecal nitrogen to dry matter consumed does not show the sharp upward trend for low intakes of dry matter that is seen with both rats and pigs. There is variation, it is true, but no such consistent tendency of the ratio in the data from human subjects as may be noticed in the rat data illustrated in Chart 5. From these data it appears that Thomas (3) and Martin and Robison (4) erred more in considering all of the metabolic nitrogen of human feces to be constant than those (9) who made the same mistake with regard to rat feces, for in the latter case there is at least a fraction of the metabolic nitrogen which is constant.

The opinion stated by Mendel and Fine (16) that, "Fasting feces are in great part derived from retained fecal matter, resulting from food immediately preceding the period of inanition" is in accord with these findings with human subjects. This viewpoint is also supported by the observations of Paton and Stockman (14) and Benedict (15) that in 30 and 31 day human fasts, respectively, no feces were excreted.

The variation of the metabolic nitrogen with high and low food intakes, the presence of a constant and varying fraction in rat and pig feces, suggest a resemblance to the findings of Folin (30) with regard to the endogenous nitrogen of the urine with high and

<sup>4</sup> In these computations, the surface areas of the rats were computed by Lee's formula (see p. 265), and those of the pigs by Brody's formula,  $S = 0.097W^{0.633}$ .

low protein intakes. The fact that there may be a qualitative difference between the constant and the varying fractions of the metabolic nitrogen (as with the endogenous nitrogen) offers an attractive problem for further research. A small amount of work on the bacterial content of the metabolic nitrogen of rats seems to suggest that this part is associated with the varying fractions.<sup>5</sup>

#### SUMMARY

1. With rats and pigs the metabolic nitrogen of the feces may be divided into two fractions: a digestive fraction which varies directly with the quantity of food dry matter consumed; and a constant fraction which is probably of true excretory origin.

2. In the case of rats, the constant fraction of the metabolic fecal nitrogen is definitely related to body size, and more closely to body surface than to body weight. It amounts to about 240 mg. per sq. m. of body surface. For it, the term endogenous nitrogen of the feces is suggested.

3. With swine the constant fraction of the metabolic fecal nitrogen has not been clearly related to body size, probably because of the smaller number of subjects and their smaller variation in weight. However, the size of this fraction averages very nearly the same for pigs as for rats when expressed to the sq. m. of body surface; *i.e.*, 222 mg.

4. With human subjects there seems to be no constant fraction of the metabolic fecal nitrogen, all of it varying in proportion to the intake of dry food.

5. The ratio of metabolic fecal nitrogen to food dry matter consumed is very nearly constant with rats and pigs if the food intakes are not so low as to induce losses in body weight. With humans, the ratio is not at all affected by variation in food intake. Hence, for use in the estimation of the true digestibility of proteins and of their biological values this ratio is valid for rats, pigs, and humans under all conditions favorable to the most accurate determination of biological values by the Mitchell method.

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# ON THE PLANT GROWTH HORMONE PRODUCED BY RHIZOPUS SUINUS

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Since it was first discovered that cell elongation in the *Avena* coleoptile is controlled by a hormone, our understanding of the nature and rôle of this substance has progressed considerably. Apart from the elucidation of its functions in promoting growth, tropisms, and other reactions of the plant, the chemical nature of the substance has been extensively studied. The active substance produced by cultures of the mold *Rhizopus suinus* was shown by Nielsen (1930) to be ether-soluble, and by Dolk and Thimann (1932) to be an unsaturated organic acid, decomposed by strong acids but not by alkalis, and readily inactivated by oxidation. Its dissociation constant, as measured by Dolk and Thimann, is  $10^{-4.76}$ . Previously, Went (1928) had shown the molecular weight of the active substance in *Avena* coleoptiles to be about 376. The active substance in human urine was isolated by Kögl and Haagen-Smit (1931) and by Kögl, Haagen-Smit, and Erxleben (1933), and shown to be an acid,  $C_{17}H_{28}(OH)_3COOH$  (auxin A), whose lactone is also active, while from malt these workers later isolated (1933) a ketohydroxy acid,  $C_{17}H_{28}O(OH)COOH$  (auxin B), which had the same activity per unit weight.

On account of the rather small amount of substance available from *Rhizopus* cultures, and also since the bulk of the partially purified product was lost through spontaneous inactivation (see section, "Concluding stages"), the chemical investigation of the active substance, begun earlier, was dropped. However, the many experiments on purification which had meanwhile been carried out showed that the active substance from *Rhizopus* did not behave in quite the same way as that from urine.



Recently, however, it was shown by Kögl, Erxleben, and Haagen-Smit (1934) that there is in urine a second active substance, identical with  $\beta$ -indolylacetic acid, and Kögl and Kostermans (1934) showed that the molecular weight of the substance produced by *Aspergillus* and by *Rhizopus* is that of  $\beta$ -indolylacetic acid rather than that of the  $C_{18}$  compounds.

Since preparations from *Rhizopus* have been extensively used for physiological work, both in this laboratory and elsewhere, the exact nature of the active substance is of considerable interest. The present paper will give evidence that the active substance produced by *Rhizopus suinus* is in fact  $\beta$ -indolylacetic acid. Identification by the preparation of derivatives and by mixed melting points with the pure synthetic substance was not possible on account of the small amount of material available. Nevertheless, the evidence given below is fairly conclusive. The method of purification, since it differs to some extent from that adopted by Kögl and his coworkers, will also be outlined. Finally, it will be shown that some of the peculiar conditions previously found to be necessary for the production of this growth substance (Thimann and Dolk, 1933) find a simple explanation on this basis.

#### *Purification of Active Substance from Rhizopus suinus*

The conditions necessary to obtain maximum yields of active substance from *Rhizopus suinus* were studied by Bonner (1932) and by Thimann and Dolk (1933). The organism has its temperature optimum at 35–37°, and its growth is inhibited by high acidity. It was found that high yields of growth substance could only be obtained from media containing peptone, and further that certain peptones only were effective. On ammonium salts the yield was only about one-tenth of that on the peptone media. The yield was also proportional to the extent to which the culture was aerated.

By observing these conditions, *i.e.* by growing the mold in a medium containing 1 per cent of the effective peptone (Witte's) and 2 per cent of dextrose, with salts, at 35°, and aerating at the optimum rate of about 25 liters of air per hour, yields of from 100 to 200 units of growth substance per cc. were obtained (*cf.* Thimann and Dolk, 1933).

Since 1 mg. of pure  $\beta$ -indolylacetic acid is equal to about  $3 \times 10^4$

of our volume units, it follows that 200 units per cc. is  $6.7 \times 10^{-4}$  mg. per cc., and since 1 cc. of the medium contains 31 mg. of solids, we have a concentration of the active substance of about 1 part in 50,000 of solids. Correspondingly, about 1500 liters are needed to give a gm. of the active substance, as against 9400 liters quoted by Kögl for *Rhizopus reflexus*.

It is worthy of note that the *Avena* test method as carried out in this laboratory does not give the large variations of several hundred per cent found by the Dutch workers, and ascribed by them to a periodic change of some kind in climatic conditions. At Pasadena, on the contrary, results from day to day are constant within about 10 to 20 per cent, tests being always carried out at about the same time of day; there may, however, be some variation over long periods. This constancy of the test enables quite small losses in activity to be determined.

*Preliminary Extractions.*—The mold was grown in a hot room in large vessels under the above conditions. About 140 liters of the medium were obtained altogether, and this was worked up in several lots. After being filtered from mycelium and spores, it was evaporated under diminished pressure to 1 to 2 per cent of its volume, filtered from precipitated peptone, etc. (Precipitate A), acidified to pH 3, and extracted about seven times with half its volume of peroxide-free ether each time. From the extracts (B and C) the ether was evaporated off and the residue taken up in hot water. On cooling, a precipitate of wax-like substances separated; this was reextracted with warm chloroform (Extract E) and the active substance so obtained brought back into water and added to the main aqueous extract.

On account of the relatively small amount of active material available an attempt was made to carry out these processes quantitatively; *i.e.*, without loss of activity. It was found that the original activity could be checked to within 1 per cent (see Table I), while 90 per cent of the original growth substance was obtained in the combined Extracts B, C, and E.

*Fractional Extraction at Controlled pH*—The aqueous solution from the extractions, containing in all 21.9 million units, was evaporated, filtered from a bulky inactive precipitate, and brought to pH 4.80 electrometrically. Another precipitate separating at this point carried down considerable activity, and it was, there-

fore, after washing, redissolved in hot water and worked up with  $\text{CHCl}_3$ , the  $\text{CHCl}_3$  extract being taken up in water with considerable loss of activity and added to the main fraction. The solution, containing now 15.97 million units, was extracted five times with an equal volume of  $\text{CHCl}_3$  each time, since this procedure should give 88 per cent extraction of an acid of dissociation constant  $10^{-4.75}$  and partition coefficient between  $\text{CHCl}_3$  and water of 1.5. The extract contained 12.58 millions, and the aqueous residue 2.90 millions; *i.e.*, 81.3 per cent was extracted. The residue, containing citric, oxalic, and other stronger acids, was discarded. The  $\text{CHCl}_3$  extract was shaken five times with half of

TABLE I  
*Quantitative Procedure in Preliminary Extractions*

Solution	Total activity <i>growth substance units</i> $\times 10^4$
Original medium.....	1040
A. Peptone ppt.....	29
B. First 3 ether extracts (filtered).....	614
C. Further 4 " ".....	79
D. Extracted liquid.....	66
E. $\text{CHCl}_3$ extract of wax-like ppt. from ether extracts.....	241
Total.....	1029
" in extracts (B + C + E).....	934 = 90%
Loss of active substance.....	1%

its volume each time of 0.5 M  $\text{NaHCO}_3$  solution, which extracted 12.26 millions or 98 per cent of the activity; the alkaline solution was reacidified and extracted repeatedly with fresh  $\text{CHCl}_3$ . The use of  $\text{CHCl}_3$  for these extractions was preferred to ether on account of the extreme sensitivity of the active substance to traces of peroxide in the ether. Although freshly distilled before use, the  $\text{CHCl}_3$  no doubt developed traces of acid on keeping, and this probably accounts for the inactivation of growth substance in  $\text{CHCl}_3$  solution which was later observed. Traces of alcohol were of course always added.

*Treatment with Organic Solvents, Etc.*—The extracts were evaporated to dryness (4.2 gm.) and extracted repeatedly with boiling

petroleum ether, b.p. 40–60°. The extracts contained 0.49 million units in 565 mg. and were discarded. The insoluble residue was extracted with warm ligroin, which removed a further small amount of inactive substance. Many other solvents were tried but gave little increase in purity.

Precipitation of the active substance as lead salt gave unsatisfactory results. Only a small part of the substance was precipitated by addition of basic lead acetate in alcoholic solution, and in addition considerable inactivation occurred, from 30 to 50 per cent of the activity being lost in the trial experiments. This was traced to the use of warm acid solution for removal of lead with  $\text{H}_2\text{S}$ , the active substance being readily decomposed by warm dilute acid (*cf.* below). Even when this procedure was avoided by extracting precipitate and filtrate directly with ether, the lead precipitation gave no increase in purity and was therefore abandoned.

At this stage the procedure of Kögl, Haagen-Smit, and Erxleben (1933), *i.e.* conversion of the acid to a lactone by boiling with 1 per cent  $\text{HCl}$  in methanol, was tested, but was also found to cause considerable inactivation. Since, however, the product showed no tendency to crystallize, it was decided to attempt distillation.

*Fractional Distillation in Vacuo*—Contrary to the findings for auxentriolic acid, it was soon found that this active substance could be distilled as the free acid almost without loss, in a high vacuum. After numerous trials a still of the Hickman type was adopted (*cf.* Hickman, 1932). It is designed to realize pressures below  $10^{-3}$  mm. in the still itself, and is a micromodification of those of Hickman, with the difference that condensation takes place on a removable inner tube (see Fig. 1). This tube fits into the main vessel—a sphere of 5 cm. radius—with a ground joint protected by a mercury seal, and its base is 2.5 cm. from the bottom of the bulb. The still was heated by immersing to about half its depth in a paraffin bath, the inner condenser tube being cooled with crushed ice. The side tube was connected through a liquid air trap to a two-stage mercury pump backed by an oil pump. The pressure was from  $3 \times 10^{-4}$  to  $1 \times 10^{-4}$  mm. of Hg.

With this "molecular still" the oil was distilled in quantities of about 300 mg. at a time. The procedure was to raise the bath temperature until a faint cloudiness appeared on the condenser; then to hold the bath at the same temperature or a few degrees

higher until no more substance appeared to distil. The vacuum was then released and the condenser tube removed and rinsed down with  $\text{CHCl}_3$  or acetone into a small dish. The condenser was replaced, a vacuum again established, and another fraction taken. Since the still is small, high vacuum is quickly reestablished. In all the distillations the bulk of the activity appeared in one fraction, that distilling at a bath temperature between  $95\text{--}105^\circ$ ,

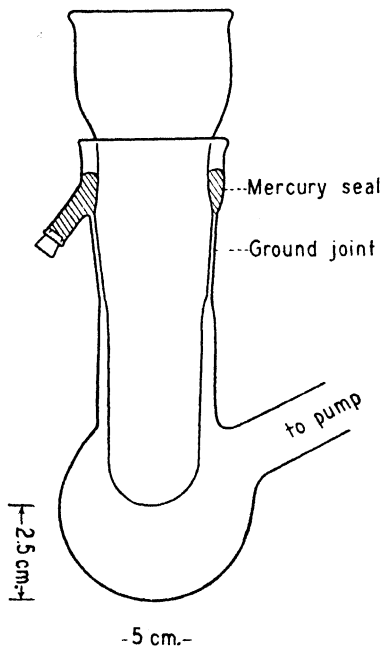


FIG. 1. Still for high vacuum fractional distillation of small quantities

and the inactivation was negligible. In the sample distillation shown in Table II, the loss of activity was 6 per cent only, while the purity was increased between 3 and 4 times in the main fraction.

The residues left above  $115^\circ$  from all twelve distillations were finally combined, reextracted with petroleum ether, and again distilled; the active distillate was, however, not combined with the other material, since its purity was lower.

*Concluding Stages*—The combined active distillates, totaling 5.0 million units, were evaporated once with  $\text{CCl}_4$  to remove the last traces of chloroform, and then extracted cautiously with  $\text{CCl}_4$  at  $0^\circ$ . The extracts on standing at  $0^\circ$  for a few days precipitated an insoluble yellow oil which was added to the other insoluble fraction. The insoluble material contained 2.5 million units in 135 mg. This was dissolved in methanol, a small amount of insoluble material being removed, and 186 mg. of brucine dissolved in methanol were added. On cooling to  $-30^\circ$  the salt was precipitated; it was filtered off and washed quickly with MeOH cooled in the same freezing mixture. The precipitate was dissolved in water, made to pH 10, and extracted with  $\text{CCl}_4$  till the extract no

TABLE II  
*Sample Distillation*

Fraction No.	Weight mg.	Activity	
		total units	units per mg.
1. $60-72^\circ$ for 40 min.....	36.7	58,000	1,600
2. $80-97^\circ$ " 10 " $95-97^\circ$ for 18 min...	48.9	900,000	18,000
3. $95-104^\circ$ " 8 " $104-115^\circ$ " 57 " .....	55.9	372,000	5,000
4. Residue above $115^\circ$ .....	135.5	159,000	1,200
Total.....	277.0	1,489,000	
Initial.....	294	1,580,000	5,400
Recovery.....	94%	94%	

longer gave the  $\text{HNO}_3$  test for brucine. The solution was then brought to pH 3 and extracted with  $\text{CHCl}_3$ . The extract, 12.8 mg., contained 1.5 million units or 117,000 units per mg. This figure, compared with 310,000 units per mg. for synthetic  $\beta$ -indolyl-acetic acid (Thimann and Koepfli, 1935) shows that the preparation was about 40 per cent as active as the synthetic product. However, the substance lost activity rapidly on being kept in  $\text{CHCl}_3$  in the dark and before it could be further studied the bulk of the activity had disappeared.

The filtrate,<sup>1</sup> which carried 900,000 units in 34.5 mg., *i.e.* was one-fourth as pure, was therefore worked up by redistillation *in*

<sup>1</sup> This solution, indicated as Br. F. (brucine filtrate), was used in the experiments on root formation of Thimann and Went (1934).

*Purification of Growth Substance from Rhizopus*

Medium, about 0.14 mg. per unit; evaporated <i>in vacuo</i> and filtered		
Ppt. discarded	Filtrate extracted seven times with ether at pH 3	
Aqueous layer discarded	Extract taken back into water and chilled	
First ppts. worked over with ether, extracts being combined with rest of active material; later ppts. discarded	Solution brought to pH 4.80 and filtered	
Ppt. discarded	Filtrate extracted five times with $\text{CHCl}_3$	
Aqueous layer discarded	Chloroform layer shaken with $\text{NaHCO}_3$	
Chloroform layer discarded	Alkaline solution acidified and extracted with chloroform; extract has 5000 units per mg.	
Aqueous layer discarded	Extract evaporated and extracted with petroleum ether	
Petroleum extract discarded	Residue extracted with ligroin	
Ligroin extract discarded	Residue transferred to molecular still	
Fractions below 95° discarded	Fraction 95–110° contains most of the activity; purity 20,000 units per mg. Combined active fractions extracted with $\text{CCl}_4$ at 0°, successively	
Extract chilled and active ppt. recovered; remainder discarded	Residue pptd. with brucine in MeOH at –30° and filtered	
Precipitate freed from brucine with $\text{CCl}_4$ ; purity 110,000 units per mg., but rapidly inactivated; lost	Filtrate therefore freed from brucine by $\text{CCl}_4$ extraction; purity 26,000 units per mg.; reextracted with petroleum ether	
Extract discarded	Residue redistilled	
Fraction 70–85°	Fractions 85–95° and 95–102°	Above 102°
Needles, m.p. 109° after recrystallization twice from acetone; inactive	Platelets, m.p. 160° (ca.) but only about 1 mg.; purity 130,000 units per mg., or $4 \times 10^{-8}$ mg. per plant unit, before recrystallization	Nothing

*vacuo*, after it had been first freed from brucine as above and again extracted with petroleum ether. Three fractions were obtained. That distilling at a bath temperature below 85° crystallized in long needles, m.p. 109°, but was almost inactive. The fractions distilling at 85–95° and 95–102° were semisolid, weighed 3.5 and 1.2 mg. respectively, and had an activity of 130,000 units per mg. They were apparently the same. On cooling, they crystallized in platelets, which on account of their small amount, could not be properly separated from the syrup. However, recrystallization from acetone was attempted. Under the microscope the crystals melted, not sharply, at 160°. Their nature will be considered below.

A summary of the purification procedure is shown in the accompanying diagram.

#### *Chemical Nature of the Active Substance*

There are thus three points of difference between the active substance from *Rhizopus* and the C<sub>18</sub> auxins from urine. The *Rhizopus* substance (1) is destroyed by warm dilute acids (this was earlier shown by Dolk and Thimann (1932)); (2) is not precipitated by basic lead acetate in neutral alcoholic solution; (3) can be distilled without destruction at about 100° *in vacuo*.

In connection with the lead precipitation, a remarkable phenomenon occurred when the same procedure was tried for comparison on a urine concentrate. This had been extracted with ether at pH 3, and the ether extracts evaporated down and freed from a quantity of crystals (hippuric acid). The extract was purified by the first three stages described above and then treated with neutral lead acetate in weakly alkaline alcoholic solution just as described by Kögl *et al.* (1933). The precipitate in this case contained one-third of the activity but at a great increase in purity. Repeated addition of lead acetate gave no further precipitate; hence the filtrate carried the bulk of the activity, but a small amount of highly active substance had been removed (Table III). The only reasonable explanation for this behavior is the presence of two different active substances. In view of the subsequent findings of Kögl *et al.* these must be auxentriolic and  $\beta$ -indolylacetic acids respectively, since only the former forms an insoluble



ble lead salt under these conditions, while the latter is always present to some extent in urine.

Examination of the active preparations showed them to contain nitrogen. In many cases the amount was so small as barely to reach the limits of detection, but it was definitely present.

*Color Reactions*—All the active preparations gave the Salkowski reaction with nitrous acid, the color having a yellowish cast compared with that given by indole. In the impure preparations the intensity of the color reactions was proportional to their biological activity. A solution containing 1 part in 10,000 of the active substance, in about 10 per cent purity, gave a color equal to that of a solution of  $\beta$ -indolylacetic acid of the same concentration. Positive tests were given with the Ehrlich and  $\text{FeCl}_3\text{-HCl}$  reactions, the latter

TABLE III  
*Lead Acetate Precipitation of Partially Purified Urine Concentrate*

Solution	Total activity	Weight	Purity
	<i>growth substance units <math>\times 10^3</math></i>	<i>mg.</i>	<i>units per mg.</i>
Ppt. 1.....	13.5	9.6	14,000
" 2.....	5.5	4.0	13,500
" 3.....	3.4	9.0	3,800
" 4.....	1.5		
Filtrate .....	28.0	92.1	3,040

of which is the more specific (*cf.* Frieber, 1922). The intensity of these tests was also strictly proportional to the activity.

The crystalline distillate, Fraction II of the redistilled brucine filtrate, which was evidently the active substance in only slightly impure state, gave positive tests in all three reactions and must therefore certainly be an indole acid derivative. Since indole, indolecarboxylic acid, indolepropionic acid, and tryptophane are all inactive, the substance must be either indoleacetic acid, or, what is very improbable, another unknown indole acid of the same activity.

*Vacuum Distillation*—Since the active substance distilled in a high vacuum at about  $100^\circ$  without much loss of activity, the same treatment was given to some synthetic  $\beta$ -indolylacetic acid, prepared by Majima and Hoshino's method (1925). In the mo-

lecular still described above no distillation occurred below 96°, but at a bath temperature of 99–103°, the substance distilled rapidly and quantitatively; 1.2 mg., activity 310,000 units per mg., yielded 1.2 mg. of distillate, activity 224,000 units per mg. The behavior is thus identical with that of the *Rhizopus* substance and different from that of the urine auxins.

*Sensitivity to Acid*—A dilute solution of pure  $\beta$ -indolylacetic acid was treated with 0.5 N HCl for 30 minutes at 100°. The activity before treatment was 99.2 and 24.8 units per cc.; after treatment, 3.5 and 0 units per cc., respectively. The destruction is thus practically complete.

*Melting Point*—Little emphasis can be placed on this property, since the crystals could not be satisfactorily separated from the syrup, but the melting point of 160° observed is close to that of 164.5° of synthetic  $\beta$ -indolylacetic acid.

*Loss of Activity on Keeping*—The loss of activity of the purified *Rhizopus* growth substance in solution is paralleled by the loss of activity of synthetic  $\beta$ -indolylacetic acid, which proceeds steadily, though apparently not so rapidly as that of auxin A. The following results were obtained with an aqueous solution of  $\beta$ -indolylacetic acid in the dark at 0°.

Day	Activity
	units $\times 10^3$ per cc.
0	53.2
3	40.8
7	35.2
17	16.2
29	9.4

#### *Conditions for Production of Active Substance by Rhizopus*

The principal findings previously reported (Thimann and Dolk, 1933) were as follows: (1) On peptone media, the yield of growth substance depends on specific substances in the peptone; these were almost completely absent from the Merck product but present in the Witte product. This mysterious substance is clearly *tryptophane*, whose presence in peptones is known to vary with their history and origin. Qualitative tests on samples of the peptones used in that study show that the Merck product used gives

almost a negative Adamkiewicz reaction in 1 per cent solution; *i.e.*, its tryptophane content is extremely low. The Witte peptone, on the other hand, gives a strong test in 1 per cent solution, even stronger than that given by so called tryptophane broth preparations. Further, the "precursor" substance was shown to be reduced in amount by repeated autoclaving. (2) The yield of growth substance is proportional to the extent to which the culture is aerated. This follows from consideration of the reaction tryptophane  $\rightarrow$   $\beta$ -indolylacetic acid, as carried out by microorganisms:



The reaction is thus a typical oxidative deamination. The mold is also capable of oxidizing the substance further, as is shown by the fall in activity of the medium after 10 days at 35°.

Both these findings are thus satisfactorily explained.

Further, Boysen-Jensen (1932) found that, with *Aspergillus*, tryptophane and the 6-carbon-containing amino acids lysine, leucine, tyrosine, and phenylalanine could be converted to growth hormone. The relationship between these compounds and  $\beta$ -indolylacetic acid is, as pointed out by Kögl and Kostermans (1934), obvious.

It may be added that, as previously reported (Thimann and Went, 1934) the activity in growth promotion was paralleled in all preparations by activity in promoting root formation, root formation being determined by the method of Went (1934). Since the activity for both functions remained approximately proportional up to the final stages of purification, the conclusion was drawn that the two hormones are either identical or very closely related. However, the fact that the crystalline  $\text{C}_{18}$  auxins and the synthetic  $\beta$ -indolylacetic acid are all about equally active in promoting root formation proves that the two hormones are identical. The synthetic substance has an activity varying between about 40,000 and 280,000 root units per mg.

#### SUMMARY

1. The purification of the plant growth-promoting substance produced by cultures of *Rhizopus suinus* is described.

2. Although the preparation of derivatives could not be carried out on the small amount of substance available, it is shown that there is no doubt that the active substance is identical with  $\beta$ -indolylacetic acid. This identity is supported by specific color reactions, the melting point (approximate), distillation temperature, and various chemical properties, as compared with those of the synthetic substance.

3. The mechanism of the production of the active substance from peptones by the mold is reviewed, and showed to be largely explained by this finding.

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# THE METHYLGLYCOSIDES OF THE NATURALLY OCCURRING HEXURONIC ACIDS\*

## IV. POLYGALACTURONIC ACID-METHYLGLYCOSIDES DERIVED FROM EHRLICH'S "PEKTOLSÄURE" AND "PEKTOLACTONSÄURE"

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In a recent communication from this laboratory a procedure was described for the isolation of a group of esterified galacturonic acid polymers through the alcoholysis of citrus polygalacturonide (1). The esters were procured as their methylglycosides by subjecting the polygalacturonide,  $(C_6H_8O_6)_n$ , to the action of dry hydrogen chloride in absolute methyl alcohol. It was shown that the major portion of this group consisted of a homogeneous fraction containing 8 to 10 galacturonic acid units in glycosidic linkage. This value was therefore considered to represent a minimum size for the structural unit of citrus pectin. At about the same time Meyers and Baker, through totally different methods, reached similar conclusions concerning the molecular size of the basic structural unit contained in pectin (2).

Ehrlich's extensive studies on the pectins had, on the other hand, led to different deductions (3-5). He concluded that the basic unit of the pectin molecule is a 4-membered ring structure,  $C_{20}H_{28}O_{16}$   $(COOH)_4 \cdot H_2O$ , called *Pektolsäure*, from which an open

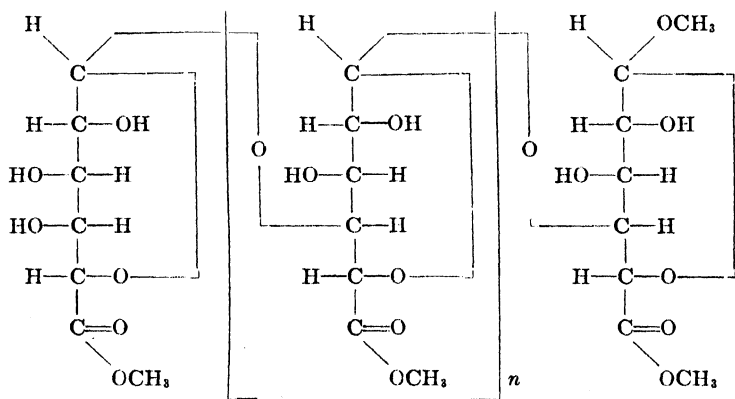
chain modification,  $C_{20}H_{28}O_{16} \left( \begin{array}{c} \text{CO} \\ \diagup \quad | \\ \text{O} \end{array} \right) (COOH)_3$ , called *Pektolactonsäure*, could be obtained by the opening of one of the saccharide linkages. The discrepancy existing between Ehrlich's conclusions

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and the findings in this laboratory led us to investigate the action of the  $\text{HCl-CH}_3\text{OH}$  reagent on his *Pektolsäure* and *Pektolactonsäure* preparations.

The action of the  $\text{HCl-CH}_3\text{OH}$  on both preparations was similar to that previously reported for citrus polygalacturonide (1, 6). The extent of alcoholysis, however, varied considerably. With *Pektolsäure*, from 25 to 35 per cent was dissolved by the reagent, whereas with the *Pektolactonsäure*, the soluble fraction amounted to 80 to 95 per cent.<sup>1</sup> From the soluble portions,  $\alpha$ -methyl-*D*-galacturonide methyl ester monohydrate ( $\text{C}_8\text{H}_{14}\text{O}_7 \cdot \text{H}_2\text{O}$ ) was isolated in yields comparable to those reported by Morell and Link (6). The portions of the *Pektolsäure* and *Pektolactonsäure* preparations which resisted solution in the  $\text{HCl-CH}_3\text{OH}$  reagent proved to be extremely inert to further treatment in the same medium, a property also exhibited by the residue from citrus polygalacturonide (1). The insoluble residues were purified by precipitation from aqueous solution with ethyl alcohol. Analysis of the residues has shown that they are practically identical to the polygalacturonic ester glycosides,  $\text{C}_6\text{H}_8\text{O}_6\text{COOCH}_3 \cdot (\text{C}_6\text{H}_7\text{O}_4\text{COOCH}_3)_n \cdot \text{C}_6\text{H}_7\text{O}_5 - (\text{OCH}_3)\text{COOCH}_3$ , previously obtained (1). The only differences observed were slight variations in the number of galacturonic acid units contained in the large molecule. The values for  $n$  of the formula varied between 7 and 10 in the polyglycoside obtained



<sup>1</sup> Under the same conditions about 50 per cent of the citrus polygalacturonide went into solution (1).

from the *Pektolsäure* preparations. The corresponding values for  $n$  in the polyester from the *Pektolactonsäure* preparations were 8 to 10; whereas in the residue from the citrus polygalacturonide,  $n$  varied between 6 and 8 (1). The values for  $n$  were determined by methoxyl analyses of the polyester glycosides, their sodium and barium salts, and their free acids.<sup>2</sup> The validity of this method for ascertaining the molecular size of these fractions has already been discussed ((1) p. 3).

From these observations it is difficult to agree with Ehrlich that the only difference between *Pektolsäure* and *Pektolactonsäure* is that the former is a 4-membered galacturonic acid ring, whereas the latter is the *open chain* structure. Since Ehrlich's preparations give rise to polymers containing approximately 10 condensed galacturonic acid units, it appears that they are not tetragalacturonic acid anhydrides. The fact that on alcoholysis the *Pektolsäure* preparations yielded 70 per cent of a resistant polygalacturonide containing approximately 10 galacturonic acid units, whereas the *Pektolactonsäure* yielded only 12 per cent of the same residue, is evidence that these preparations differ radically in molecular size. Their different solubilities in water is also in accordance with this view. Since the extent of alcoholysis during the prolonged HCl-CH<sub>3</sub>OH treatment is not known, it may only be concluded that the *minimum* size of the galacturonic acid complex in *Pektolsäure* involves approximately 10 galacturonic acid units. As Ehrlich has obtained similar preparations from the pectins of a great many tissues (7), our observations concerning their polygalacturonic structure may be generally applied to all pectic substances.

#### EXPERIMENTAL

The polygalacturonic ester glycosides, barium salts, sodium salts, and free acids were prepared and characterized by the methods previously described (1). The analytical values cited in Tables I to V represent the averages of several preparations. The differences between methoxyl analyses for check determinations did not vary more than 0.1 per cent in any case. Since the methoxyl contents varied within the limits required for polymers

<sup>2</sup> With the insoluble methylated residue from *Pektolactonsäure* the analyses were confined to the polyester glycoside.



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of 8 to 12 galacturonic acid units, theoretical values cited in Tables I to V have been calculated for a molecular size of 10 units.

*Preparation of Ehrlich's Pektolsäure and Pektolactonsäure* (4)—Citrus and apple pectins<sup>3</sup> were purified by three extractions with hot 70 per cent alcohol. The uronic anhydride contents were 73 and 56 per cent respectively. On heating solutions of these pectins in 5 per cent hydrochloric acid at 85–90°, a slimy precipitate of *Pektolsäure* gradually separated. After 4 hours the solutions were filtered and the precipitates washed with water. The filtrate

TABLE I

*Analytical Values Observed for Pektolsäure and Pektolactonsäure Prepared from Citrus and Apple Pectin*

Source	Rotation	Titration equivalents	Methoxyl	Iodine consumption
	degrees	cc. 0.1 N NaOH per gm.	per cent	cc. 0.1 N I <sub>2</sub> per gm.
Citrus pectin				
<i>Pektolsäure</i> .....	+286.0	55.0	1.5	4.27
<i>Pektolactonsäure</i> .....	+250.0	54.3	1.6	24.0
Apple pectin				
<i>Pektolsäure</i> .....	+270.0	53.4	1.3	2.9
<i>Pektolactonsäure</i> .....	+237.0	55.9	0.9	24.3
Ehrlich's values				
<i>Pektolsäure</i> .....	+290.0*	55.4†		1.9‡
<i>Pektolactonsäure</i> .....	+250.0*	56.8†		26.3‡

\* Cited by Ehrlich in summary form ((5) p. 528).

† Theoretical titration equivalents ((5) p. 528).

‡ (3) p. 2012.

and washings were concentrated under reduced pressure and poured into 5 volumes of ethyl alcohol. Snow-white amorphous precipitates of Ehrlich's *Pektolactonsäure* immediately separated. The *Pektolsäure* was purified by reprecipitation with acid from a large volume of water. The preparations were dried at 100° in a vacuum oven for 10 hours. The yields from citrus pectin were 25

<sup>3</sup> We wish to express our appreciation to the Research Department of the California Fruit Growers Exchange, Ontario, California, for the citrus pectin, and to the Research Department of the General Foods Corporation, Battle Creek, Michigan, for the apple pectin used in this investigation.

per cent for the purified *Pektolsäure* and 9 per cent for *Pektolactonsäure*; with apple pectin the corresponding yields were 24 per cent and 8 per cent. In Table I the analytical constants observed are cited. Ehrlich's values for his preparations are also included for comparison.

*Methylglycoside of Polygalacturonic Methyl Ester*—15 gm. of each of the preparations described above were subjected to the

TABLE II  
*Methylglycoside of Polygalacturonic Methyl Ester*

Methylation residue from	Yield	Meth-oxyl	Rotation in H <sub>2</sub> O, c = 1.5-2.0	Iodine consumption	Saponification equivalent
	per cent	per cent	degrees	cc. 0.1 N I <sub>2</sub> per gm.	cc. 0.1 N NaOH per gm.
Citrus pectin					
<i>Pektolsäure</i> .....	66.3	17.80	+221.7	2.61	48.86
<i>Pektolactonsäure</i> .....	16.0	17.34	+198.1	2.12	49.13
Apple pectin					
<i>Pektolsäure</i> .....	73.3	17.32	+218.7	1.90	49.21
<i>Pektolactonsäure</i> .....	8.9	17.36	+201.0	1.25	49.35
Theory.....		17.67		0.0	51.78

TABLE III  
*Methylglycoside of Sodium Polygalacturonate*

Source	Yield	Meth-oxyl	Sodium	Rotation in H <sub>2</sub> O, c = 1.5-2.0	Iodine consumption
	per cent	per cent	per cent	degrees	cc. 0.1 N I <sub>2</sub> per gm.
Citrus pectin, <i>Pektolsäure</i> .....	90.0	1.47	11.97	+223.4	0.52
Apple pectin, <i>Pektolsäure</i> .....	90.0	1.36	11.62	+223.8	0.48
Theory.....		1.54	11.43		0.0

HCl-CH<sub>3</sub>OH treatment formerly described (1). The analytical constants for the polyester glycosides, after purification, are cited in Table II. The yield of crystalline  $\alpha$ -methyl-*D*-galacturonide methyl ester monohydrate, obtained from the alcoholic solutions, was  $30 \pm 5$  per cent, which is in good agreement with the original findings (6).

*Methylglycoside of Sodium Polygalacturonate*—2.0 gm. samples

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of the polyester glycoside were used for the preparation of the sodium salt. The analytical results are summarized in Table III.

*Methylglycoside of Barium Polygalacturonate*—3.0 gm. portions of the polyester were used to prepare the barium salts. In Table IV the observed analytical constants are given.

*Methylglycoside of Polygalacturonic Acid*—The polygalacturonic acid was prepared from its ester. 3.0 gm. portions of the purified residues were used. The results are summarized in Table V.

Since the original *Pektolsäure* preparations were not free of methoxyl, it was necessary to study their behavior when carried

TABLE IV  
*Methylglycoside of Barium Polygalacturonate*

source	Yield	Methoxyl	Barium
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Citrus pectin, <i>Pektolsäure</i> .....	95.0	1.48	26.32
Apple pectin, <i>Pektolsäure</i> .....	95.0	1.28	26.50
Theory.....		1.26	27.8

TABLE V  
*Methylglycoside of Polygalacturonic Acid*

Source	Yield	Methoxyl	Rotation in H <sub>2</sub> O, <i>c</i> = 1.5-2.0	Iodine consump- tion
	<i>per cent</i>	<i>per cent</i>	<i>degrees</i>	<i>cc. 0.1 N I<sub>2</sub> per gm.</i>
Citrus pectin, <i>Pektolsäure</i> .....	67.0	1.31	+261.4	1.41
Apple pectin, <i>Pektolsäure</i> .....	65.0	1.36	+259.0	0.93
Theory.....		1.73		0.0

through the alkali-acid procedure for making the free polyacid from its ester ((1) p. 10). 5.0 gm. samples of *Pektolsäure*, prepared from both citrus and apple pectin, were suspended in 40 cc. of water and 30 cc. of *N* NaOH were added slowly with stirring. The procedure for preparing the polyacid from its ester was then repeated in detail. The methoxyl content of the citrus *Pektolsäure*, after two such treatments, fell from an original value of 1.60 per cent to 0.30 per cent, where it remained constant. The methoxyl content of the apple *Pektolsäure*, when similarly treated, fell from 1.29 per cent to 0.35 per cent.

## SUMMARY

1. The action of dry hydrogen chloride in absolute methyl alcohol on Ehrlich's *Pektolsäure* and *Pektolactonsäure* preparations, obtained from both citrus and apple pectin, has been studied.

2. When refluxed for 90 hours, about 30 per cent of the *Pektolsäure* and 90 per cent of the *Pektolactonsäure* were dissolved.

3. In each case the insoluble residue consisted mainly of an esterified polygalacturonide in the form of its methylglycoside. Analysis of this polyester, as well as its sodium salt, barium salt, and free acid, has shown that it contained approximately 10 galacturonic acid units.

4. Ehrlich's belief that his *Pektolsäure* and *Pektolactonsäure* preparations are definite compounds containing 4 galacturonic acid units is not in harmony with the results obtained.

We are indebted to our colleague, Dr. Sam Morell, for the interest and constant counsel that he contributed to this investigation.

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## A STUDY OF THE ORGANIC ACID-SOLUBLE PHOSPHORUS OF THE ERYTHROCYTES OF VARIOUS VERTEBRATES

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Hoffman (1925) isolated from blood adenylic acid, the presence of which had been indicated by the work of Jackson (1923, 1924). Lohmann (1928, *a*) found in muscle a phosphorus compound easily hydrolyzed by normal acid, and designated it pyrophosphate. The presence of easily hydrolyzable organic phosphorus in blood had already been recognized (Zucker and Gutman, 1922-23), and the quantity of this so called "pyrophosphate" was determined in the blood of various animals (Lohmann, 1928, *b*); Barrenscheen and Vasarhelyi, 1930; Bomskov, 1932). When it was learned that muscle adenylic acid (Embden and Zimmermann, 1927) and "pyrophosphate" were actually present in combination as adenosine triphosphate (Fiske and Subbarow, 1929; Lohmann, 1929), it became a matter of interest to determine whether the adenylic acid and hydrolyzable phosphorus of blood were likewise present in combination with each other. Barrenscheen and Filz (1932) attempted to isolate the silver salt of adenosine triphosphate from human blood and obtained a low yield of a substance differing in composition from the adenosine triphosphate secured from muscle.

With the purpose of learning whether or not the adenine nucleotide and hydrolyzable phosphorus were present in blood in the proportion required if they were combined as adenosine triphosphate, we determined the quantities of these two components in the blood of twenty-four species of animals. Since the completion of our experiments Fiske (1934) has announced the isolation of adenosine triphosphate in nearly quantitative yield from rabbit blood and found it indistinguishable from that prepared from muscle. Although Fiske's experiments give direct evidence that all the adenylic acid of rabbit blood is present as adenosine tri-

phosphate, it remains to be determined whether all of the easily hydrolyzed organic phosphorus is also so combined. Our data therefore become of interest in regard to the relative proportions of adenylic acid and hydrolyzable phosphorus as well as species differences in the organic phosphorus compounds of the erythrocytes.

### Methods

Blood was obtained from heart or vein<sup>1</sup> directly from the animals or from the slaughter-house, defibrinated by gentle stirring, and then pipetted at once into trichloroacetic acid in order to prevent breakdown of organic phosphorus.<sup>2</sup> This procedure was invariably carried out at the place of bleeding. Specimens prepared in this way at the slaughter-house or outside the city (*e.g.* camel blood) reached the laboratory within an hour, and in these cases the flasks were surrounded with ice during the period of transport. The determination of inorganic and hydrolyzable phosphorus was begun at once, and that for nucleotide before the end of the same day. The hematocrit was determined in a separate portion of the defibrinated blood in special 10 cm. graduated capillary tubes, and the corpuscle composition calcu-

<sup>1</sup> The hyena, jackal, wolves, and monkey were anesthetized by intraperitoneal injection of amytal. Other animals were bled without anesthesia.

<sup>2</sup> In earlier experiments, which had to be discarded, separated corpuscles were analyzed. They were weighed in volumetric flasks and laked with water, but the breakdown of organic phosphorus was so rapid that the values for inorganic phosphorus became much higher than the usual normal for whole blood, especially in the experiments with turkey, goose, and pig. This was due partly to slow autolysis of the easily hydrolyzable phosphorus during centrifugation, but chiefly to a very rapid autolysis after laking (Barrenschæen and Braun, 1930). The loss of easily hydrolyzable phosphorus in various bloods, laked and unlaked, was found to be as follows:

Pig blood, unlaked.....	7	% loss in 1 hr.
“ “ laked.....	7	% 1 min.
“ “ “.....	33	% 1 hr. at 14°
“ “ “.....	48	% 1 “ “ 25°
Human blood, laked.....	24	% 1 “ “ 13°
Dog blood, unlaked.....	11.5	% 2½ hrs.
“ “ laked.....	59.4	% 2½ “
“ “ “.....	16.0	% 1 hr.
Turkey blood, unlaked.....	21	% 4 hrs.
“ “ laked.....	76	% 1½ “

lated from the results of the analysis of whole blood, the minute amount of organic phosphorus in serum being considered negligible for the purpose of this study.<sup>3</sup>

Total acid-soluble and inorganic phosphorus were determined by the methods of Fiske and Subbarow (1925). "Hydrolyzable" phosphorus was determined by heating at 100° in the presence of  $N$  HCl for a period of 7 minutes (Lohmann, 1928, *a*), after which the liberated inorganic phosphate was measured by the Fiske and Subbarow procedure. Nucleotide was determined by the method of Kerr and Blish (1932). All determinations were performed in duplicate or triplicate, except in a few cases where the material available was insufficient.

#### DISCUSSION

Examination of the data presented in Table I reveals a definite parallelism between the values for nucleotide and hydrolyzable phosphorus. When the averages for various species are considered, the ratio of nucleotide to hydrolyzable phosphorus is found to vary between the extremes of 0.86:2 (buffalo) and 1.30:2 (camel), but for most species lies close to 1:2, the ratio expected if the two components were in combination with each other as adenosine triphosphate. In a relatively small number of individuals the ratios varied widely from the mean, occasional values being as low as 0.53:2 (goat) and as high as 1.75:2 (ox).

Provided the divergent ratios could be satisfactorily explained, the parallelism existing between nucleotide and hydrolyzable phosphorus in most of the specimens would suggest that the two fractions were combined as adenosine triphosphate. However, more direct evidence that all the nucleotide is actually present as the triphosphate is found in the recent publication of Fiske (1934), who states that charcoal completely adsorbs the nucleotide from dilute aqueous solutions, that two-thirds of the phosphorus ad-

<sup>3</sup> Kay (1928) states that not more than traces of organic phosphorus are found in the plasma of pig, rabbit, rat, guinea pig, man, dog, horse, cat, sheep, ox, and goat. McCay (1931), however, found as much as 5 gm. per cent of organic phosphorus in the plasma of the pike. We studied chicken serum and in three specimens (each representing the mixed blood of three birds) found the average organic phosphorus to be 0.19 mg. per 100 cc.



TABLE I

*Distribution of Acid-Soluble Phosphorus in Blood of Various Vertebrates, with Molar Ratio of Nucleotide to Hydrolyzable Phosphorus*

Species	Mg. P per 100 cc. whole blood					Ratio of nucleotide to hydrolyzable P	Hematocrit	Mg. P per 100 cc. corpuscles*			
	Total acid-soluble	Inorganic	Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined		Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined
Dog ( <i>Canis familiaris</i> )	24.1	4.3	19.8	2.2	1.3	16.3	1.18:2				
	24.8	4.9	19.9	2.4	1.3	16.2	1.08:2				
	24.9	4.5	20.4	2.1	1.3	17.0	1.24:2				
	28.7	4.5	24.2	3.1	1.7	19.4	1.10:2	42.3	57.1	7.3	4.0
	16.8	2.3	14.5	1.8	0.9	11.8	1.00:2	26.0	55.8	6.9	3.5
Average	23.8	4.1	19.8	2.3	1.3	16.2	1.12:2		56.5	7.1	3.8
Wolf ( <i>Canis lupus</i> )	26.3	4.5	21.8	3.2	1.5	17.1	0.94:2	38.2	57.1	8.4	3.9
	27.3	4.1	23.2	3.3	1.5	18.4	0.91:2	40.6	57.1	8.1	3.7
Average	26.8	4.3	22.5	3.3	1.5	17.7	0.93:2		57.1	8.3	3.8
Jackal ( <i>Canis aureus</i> )	17.5	6.2	11.3	2.1	1.0	8.2	0.95:2	26.4	42.8	8.0	3.8
Hyena ( <i>Hyæna striata</i> )	5.7	2.9	2.8	0.9	0.45	1.4	1.00:2	33.2	8.4	2.7	1.4
Cat ( <i>Felis domestica</i> )	12.4	6.5	5.9	1.6	0.8	3.5	1.00:2	26.0	22.7	6.2	3.1
	11.3	6.9	4.4	1.7	0.7	2.0	0.82:2	22.9	19.2	7.4	3.1
	12.5	5.2	7.3	2.0	1.0	4.3	1.00:2	36.4	20.1	5.5	2.8
Average	12.1	6.2	5.9	1.8	0.8	3.3	0.94:2		20.7	6.4	3.0
Rabbit ( <i>Lepus cuniculus</i> )	37.0	3.6	33.4	6.3	3.8	23.3	1.21:2				
	49.6	5.5	44.1	7.2	4.2	32.7	1.17:2				
	37.3	4.2	33.1	5.5	3.1	24.5	1.13:2				
	39.1	4.7	34.4	6.3	3.2	24.9	1.02:2	42.0	81.9	15.0	7.6
	43.4	4.7	38.7	7.0	3.2	28.5	0.91:2	41.3	93.7	16.9	7.7
Average	41.3	4.5	36.7	6.4	3.5	26.8	1.09:2		87.8	16.0	7.7
Albino rat ( <i>Mus norvegicus albinus</i> )	25.8	3.2	22.6	5.4	2.7	14.5	1.00:2				
	29.2	4.8	24.4	3.7	2.0	18.7	1.08:2	48.8	50.0	7.6	4.1
Average	27.5	4.0	23.5	4.6	2.4	16.5	1.04:2		50.0	7.6	4.1

\* Calculated from hematocrit and analysis of whole blood.

TABLE I—Continued

Species	Mg. P per 100 cc. whole blood						Ratio of nucleotide to hydrolysable P	Hematocrit	Mg. P per 100 cc. corpuscles*				
	Total acid-soluble	Inorganic	Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined			Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined	
Guinea pig ( <i>Cavia domestica</i> )	26.3	3.5	22.8	2.9	1.6	18.3	1.10:2						
	29.3	6.1	23.2	3.4	2.1	17.7	1.24:2	37.7	61.5	9.0	5.6	46.9	
	29.1	6.1	23.0	2.8	1.9	18.3	1.36:2	36.5	63.0	7.7	5.2	50.1	
	23.6	3.6	20.0	3.0				31.0	64.5	9.7			
	32.2	5.3	27.8	4.0	2.3	21.5	1.15:2	40.2	69.2	10.0	5.7	53.5	
Average	28.1	4.9	23.4	3.2	2.0	18.2	1.21:2		64.6	9.1	5.5	50.0	
Man	21.3	3.8	17.5	3.4	2.2	11.9	1.29:2	35.7	49.0	9.5	6.2	33.3	
	24.1	12.9	21.2	5.8	2.8	12.6	0.97:2	45.0	47.1	12.9	6.2	28.0	
	23.5	3.4	20.1	5.1				39.6	50.7	12.9			
	20.8	4.0	16.8	3.8	2.3	10.7	1.21:2						
	25.5	3.7	21.8	5.3	2.8	13.7	1.06:2						
	25.8	3.2	22.6	5.4	2.7	14.5	1.00:2						
		3.9		6.0	3.3		1.10:2	48.1		12.5	6.9		
		2.5		4.5	2.7		1.20:2	47.4		9.5	5.7		
		2.7		6.6	3.4		1.03:2	49.6		13.3	6.8		
		3.0		6.9	3.0		0.87:2	42.0		16.4	7.1		
		3.1		5.4	2.8		1.04:2	47.7		11.3	5.9		
		3.2		4.5	2.3		1.02:2	45.0		10.0	5.1		
		2.4		5.0	2.5		1.00:2	43.3		11.6	5.8		
		3.0		5.5	2.8		1.02:2	46.5		11.8	6.0		
		2.8		5.7	2.9		1.02:2	46.9		12.2	6.2		
		2.8		6.1	3.1		1.02:2	49.6		12.3	6.2		
		2.3		5.6	2.7		0.96:2	44.9		12.5	6.0		
		2.8		5.8	3.0		1.03:2	46.4		12.5	6.5		
Average	23.5	3.1	20.4	5.4	2.8	12.2	1.05:2		48.9	12.1	6.2	30.6	
Monkey ( <i>Macacus</i> )	19.4	3.6	15.8	3.0	1.3	11.5	0.87:2	35.5	44.5	8.5	3.7	32.3	
Pig ( <i>Sus scrota</i> , var. <i>melitensis</i> )	43.2	6.1	37.1	7.8	4.0	25.3	1.03:2						
	40.4	4.9	35.5	7.5	3.4	24.6	0.91:2						
	40.7	4.6	36.1	9.2	4.3	22.6	0.93:2	37.9	95.3	24.3	11.3	59.7	
	38.0	4.3	33.7	7.7	3.7	22.3	0.96:2	36.0	93.7	21.4	10.3	62.0	
Average	40.6	5.0	35.6	8.1	3.9	23.6	0.96:2		94.5	22.8	10.8	60.9	

TABLE I—Continued

Species	Mg. P per 100 cc. whole blood					Ratio of nucleotide to hydrolyzable P	Hematocrit	Mg. P per 100 cc. corpuscles*			
	Total acid-soluble	Inorganic	Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined		Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined
Ox ( <i>Bos taurus</i> )	6.6	3.3	3.3	1.2	0.8	1.3	1.33:2	42.0	7.9	2.9	3.1
	9.0	5.1	3.9	1.3	1.1	1.5	1.70:2	42.0	9.2	3.1	3.5
	7.4	3.9	3.5	1.7	0.9	0.9	1.06:2				
	9.4	5.5	3.9	1.8	1.0	1.1	1.11:2				
	10.0	5.9	4.1	1.9	0.9	1.3	0.95:2				
	11.1	7.6	3.5	2.4	0.8	0.3	0.67:2				
	9.0	6.3	2.7	1.1	0.7	0.9	1.27:2				
	9.1	6.0	3.1	1.2	0.8	1.1	1.33:2				
	6.6	3.7	2.9	0.8	0.7	1.4	1.75:2				
	10.1	5.2	4.9	1.6	0.9	2.4	1.13:2	37.0	13.3	4.3	6.6
	8.5	5.4	3.1	1.4	0.6	1.1	0.86:2	31.2	9.9	4.5	3.5
	7.4	5.3	2.1	0.7	0.6	0.8	1.71:2	41.2	5.1	1.7	1.9
	7.8	4.3	3.5	1.5	0.6	1.4	0.80:2	39.4	9.0	3.8	3.7
Average	8.6	5.2	3.4	1.4	0.8	1.2	1.20:2		9.1	3.4	3.7
Goat ( <i>Capra hircus</i> , var. <i>syriaca</i> )	7.3	4.1	3.2	1.5	0.9	0.8	1.20:2	28.9	11.1	5.2	2.8
	12.0	8.4	3.6	1.7	0.9	1.0	1.06:2	27.6	13.0	6.1	3.6
	7.9	4.9	3.0	1.4	0.7	0.9	1.00:2				
	8.2	4.2	4.0	1.5	0.8	1.7	1.07:2				
Average	9.1	5.6	3.5	1.6	0.8	1.1	0.97:2		12.1	5.7	3.2
Sheep ( <i>Ovis aries</i> , var. <i>crassicanus</i> )	9.5	5.0	4.5	1.9	1.1	1.5	1.16:2	32.6	13.8	5.8	4.6
	9.2	5.1	4.1	1.9	1.2	1.0	1.26:2	29.5	13.9	6.4	3.4
Average	9.4	5.1	4.3	1.9	1.2	1.2	1.21:2		13.9	6.1	4.0
Buffalo ( <i>Bos bubalis</i> )	8.0	4.3	3.7	1.4	0.5	1.8	0.71:2				
	7.8	4.7	3.1	1.3	0.5	1.3	0.77:2				
	7.0	4.1	2.9	1.1	0.6	1.2	1.09:2				
Average	7.6	4.4	3.2	1.3	0.5	1.4	0.86:2				

TABLE I—Continued

Species	Mg. P per 100 cc. whole blood						Ratio of nucleotide to hydrolysable P	Hematocrit	Mg. P per 100 cc. corpuscles*			
	Total acid-soluble	Inorganic	Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined			Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined
Camel ( <i>Camelus dromedarius</i> )	19.9	5.5	14.4	1.6	1.1	11.7	1.38:2†	23.2	62.1	6.9	4.7	50.5
	21.4	4.1	17.3	1.8	1.2	14.3	1.33:2†	24.5	70.6	7.3	4.9	58.4
	16.2	3.6	12.6	1.4	1.1	10.1	1.57:2†	22.4	56.2	6.3	4.9	45.0
	17.9	4.9	13.0	2.0	1.3	9.7	1.30:2	24.1	53.7	8.3	5.4	40.0
	20.9	4.8	16.1	2.6	1.6	11.9	1.23:2	27.2	59.2	9.6	5.9	43.7
	20.2	4.9	15.3	2.9	1.7	10.7	1.17:2	22.5	68.0	12.9	7.6	47.5
	22.8	4.7	18.1	3.1	1.7	13.3	1.10:2	26.0	69.6	11.9	6.5	51.2
Average	19.9	4.6	15.3	2.2	1.4	11.7	1.30:2		62.8	9.0	5.7	48.1
Horse ( <i>Equus caballus</i> )	24.6	2.4	22.2	1.3	0.6	20.3	0.92:2	48.8	45.5	2.7	1.2	41.6
	18.0	2.1	15.9	1.1	0.6	14.2	1.09:2	33.2	47.9	3.3	1.8	42.8
Average	21.3	2.3	19.1	1.2	0.6	17.3	1.01:2		46.7	3.0	1.5	42.2
Ass ( <i>Equus asinus</i> )	14.4	1.8	12.6	0.7	0.5	11.4	1.43:2	30.1	41.8	2.3	1.7	37.8
	14.6	2.7	11.9	1.2	0.4	10.3	0.67:2	31.2	38.1	3.8	1.3	33.0
	20.2	5.4	14.8	0.8	0.5	13.5	1.25:2					
Average	16.4	3.3	13.1	0.9	0.5	11.7	1.12:2		40.0	3.1	1.5	35.4
Mule	13.9	2.5	11.4	0.9	0.5	10.0	1.11:2	30.8	37.0	2.9	1.6	32.5
	12.9	2.0	10.9	1.1	0.5	9.3	0.91:2	20.2	54.0	5.4	5.4	46.1
Average	13.4	2.3	11.2	1.0	0.5	9.7	1.01:2		45.5	4.2	2.1	39.2
Chicken ( <i>Gallus bankiva</i> )	25.5	3.2	22.3	2.6	1.6	18.1	1.23:2	22.0	101.4	11.8	7.3	82.3
	39.4	2.8	36.6	3.9	1.6	31.1	0.82:2	38.2	95.8	10.2	4.2	81.4
		2.9		4.6	2.3		1.00:2	41.9		11.0	5.5	
		2.2		2.6	1.7		1.31:2	35.0		7.4	4.9	
		1.4		2.0	1.4		1.40:2	24.0		8.3	5.8	
Average	33.0	2.5	31.5	3.1	1.7	26.7	1.15:2		98.6	9.7	5.5	83.4

† The proteins were not precipitated until blood reached the laboratory, half an hour after the specimen was taken.

TABLE I—Concluded

Species	Mg. P per 100 cc. whole blood						Ratio of nucleotide to hydrolyzable P	Hematocrit	Mg. P per 100 cc. corpuscles*			
	Total acid-soluble	Inorganic	Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined			Organic acid-soluble	Organic, hydrolyzed in 7 min.	Nucleotide	Undetermined
Turkey ( <i>Melagris gallopavo</i> )	4.2		6.4					38.3		16.7		
	44.7	4.4	40.3	7.9				40.0		19.8		
	38.8	3.3	35.5	6.0	3.7	25.8	1.23:2	37.2	95.4	16.1	9.9	69.4
	43.2	3.4	39.8	7.0	4.3	28.5	1.23:2	43.3	91.9	16.2	9.9	65.8
	35.0	3.5	31.5	4.3	2.8	24.4	1.30:2					
Average	42.1	5.4	36.7	6.4	3.9	26.4	1.22:2					
Goose ( <i>Anser domesticus</i> )	57.2	5.1	52.1	12.0				55.9	93.2	21.5		
	52.1	5.1	47.0	8.8	5.2	33.0	1.18:2	47.7	98.6	18.5	10.9	69.2
	49.8	5.4	44.4	9.2	5.3	29.9	1.15:2	40.0	111.0	23.0	13.3	74.7
Average	53.0	5.2	47.8	10.0	5.3	32.5	1.17:2		100.9	21.0	12.1	67.8
Duck ( <i>Anas boscas</i> )	45.5	6.1	39.4	8.2	4.8	26.4	1.17:2	36.2	108.8	22.6	13.3	72.9
	44.1	7.3	36.8	7.8	4.8	24.2	1.23:2	32.8	112.2	23.8	14.7	73.7
Average	44.8	6.7	38.1	8.0	4.8	25.3	1.20:2		110.5	23.2	14.0	73.3
Sea-turtle	18.6	6.8	11.8	4.4	2.4	5.0	1.09:2	27.2	43.4	16.2	8.8	18.4

sorbed by this procedure from a protein-free filtrate of rabbit blood is hydrolyzed in 15 minutes by  $N$  HCl, and that a quantity of adenosine triphosphate can be isolated equivalent to 95 per cent of the organic phosphorus adsorbed by the charcoal.

It must be noted that the one value given by Fiske for the adenosine triphosphate content of blood (6.79 mg. of P per 100 cc. of rabbit blood) is only two-thirds of the average value (9.9 mg.) we obtained for the sum of nucleotide and hydrolyzable phosphorus in the whole blood of this species. However, the absence of any value for hematocrit in his experiment makes the comparison of little value.

Among possible explanations of the variation of our ratios from the theoretical 1:2 required for pure adenosine triphosphate must

be included experimental error and the possible presence in variable quantity of relatively small amounts of non-nucleotide hydrolyzable phosphorus compounds, as well as free adenylic acid or phosphoric esters of adenosine other than the mono- and triphosphate. In blood with a low content of nucleotide and hydrolyzable phosphorus small errors of analysis produce considerable distortion of the ratio, and it is actually in the bloods with low nucleotide that the widest variation and greatest irregularity were found (ox, goat, buffalo, ass). The ratios were also irregular in camel and chicken blood. In the former the ratios indicate the presence of some nucleotide containing less than three phosphate groups. The time required for defibrinating the blood of several chickens in order to obtain a sufficiently large pooled specimen may have permitted enough autolysis to account for the irregular and high proportion of nucleotide.

In the blood of birds and the turtle also the proportion of nucleotide is somewhat in excess of that demanded for adenosine triphosphate, although no more so than in a number of specimens of mammalian blood examined. In the analysis of the blood of birds and reptiles exposure of the erythrocyte nucleus to a trichloroacetic acid concentration as high as 8 per cent may possibly result in hydrolysis of some nucleoprotein. If hydrolysis of the nucleic acid occurred, or if the nucleic acid escaped precipitation with the proteins, the proportion of nucleotide to hydrolyzable phosphorus would be appreciably increased, as actually found.

In 73 out of 96 specimens analyzed, the quantity of hydrolyzable phosphorus was found to be slightly less than twice the nucleotide phosphorus.<sup>4</sup> If Fiske's observation (that in rabbit blood the

<sup>4</sup> It is, of course, recognized that the "hydrolyzable" phosphorus fraction originates not only from a partial hydrolysis of adenosine triphosphate but also from a partial hydrolysis of the other organic phosphorus compounds of blood. It should be noted that a 7 minute hydrolysis time was used in our determinations of the hydrolyzable phosphorus, whereas according to Fiske (1934) 15 minutes are required to convert two-thirds of the phosphorus of adenosine triphosphate to orthophosphate. A study of the hydrolysis curves of the organic phosphorus in dog and human blood indicates that the inorganic phosphorus liberated in 7 minutes hydrolysis is about 100 per cent less than the desired two-thirds of adenosine triphosphate phosphorus, after the partial hydrolysis of other compounds is corrected for. However, even after addition of 10 per cent to the values for hydrolyzable phosphorus this fraction remains somewhat less than twice the value for nucleotide phosphorus in 60 per cent of the blood specimens.

nucleotide is present entirely as adenosine triphosphate) should be found to apply to all species, then our results would indicate that the organic phosphorus hydrolyzed in 7 minutes is entirely accounted for by adenosine triphosphate in 76 per cent of the specimens examined. In only a few of the remaining cases is the excess of hydrolyzable phosphorus significant as far as quantity is concerned. In these specimens the extra phosphate must have its origin in one or more non-nucleotide organic compounds which are at least partially hydrolyzed by the treatment used. This surplus of hydrolyzable phosphorus was found in three out of four specimens of pig blood, also in the blood of two wolves, one jackal, one monkey, and a few scattered individuals differing from others of the same species.

#### *Species Differences*

The content of nucleotide and hydrolyzable phosphorus varies greatly in the blood of various species. The averaged values for nucleotide phosphorus lie between a minimum of 1.4 mg. per 100 cc. of corpuscles (hyena) and the maximum of 14.0 (duck), while the hydrolyzable phosphorus varies from 2.7 to 23.2 mg. per 100 cc. of corpuscles. The proportion which the sum of these two fractions bears to the total organic acid-soluble phosphorus is, however, greatest in the case of the goat, where it represents 73.5 per cent of the organic phosphorus. The undetermined fraction of organic phosphorus is at a minimum in the blood of the ox, goat, sheep, and buffalo, and at its highest concentration in the corpuscles of bird, rabbit, and pig.

Related animals occasionally show marked differences in blood composition. The camel's blood contains far more organic phosphorus, particularly the undetermined fraction, than that of the other ruminant artiodactyls. The chicken has a much lower content of nucleotide and hydrolyzable phosphorus than the turkey, goose, and duck, but a higher content of the undetermined fraction. Among the carnivores also, considerable variation in blood composition is found. The dog, wolf, and jackal are similar with respect to the level of nucleotide and hydrolyzable phosphorus, whereas the cat has somewhat less and the hyena a far lower content. In the undetermined fraction also great differences are noted, the corpuscles of the dog and wolf containing 10 times as much as those of the hyena and 4 times as much as those of the cat.

*Organic Phosphorus and Glycolysis*

Engelhardt and Ljubimowa (1930) found that the extent of glycolysis in the blood of different species varied in the same direction as the amount of organic phosphorus broken down during autolysis of blood.<sup>2</sup> Barrenscheen and Braun (1930) pointed out that hemolysis and the resulting breakdown of "pyrophosphate" inhibit glycolysis. When the loss of "pyrophosphate" is reinterpreted as autolysis of adenosine triphosphate, the loss of glycolytic power is better understood, in view of the known rôle of this substance in the fermentation of carbohydrates. Meyerhof (1932) has suggested that the loss of glycolytic power in hemolyzed blood is more likely due to deaminization of adenylic acid than to loss of the organic "pyrophosphate," since hemolysates prepared at 0° may lose their glycolytic power on warming to 37°, but suffer no diminution in the hydrolyzable phosphorus fraction.

Barrenscheen and Vasarhelyi (1930) claim to have shown that the pyrophosphate content of the blood of different species varies in the same direction as the glycolytic power, with the exception of pig blood. They used for this comparison an arrangement of species suggested by Loeb (1913) in order of increasing glycolytic power. This arrangement, together with the values found by Barrenscheen and Vasarhelyi (1930) for the organic phosphorus hydrolyzed in 7 minutes, in mg. per 100 cc. of whole blood, is as follows: pig<sup>5</sup> (7.83) < sheep < ox (1.48) < dog (2.77).

This seems rather meager information on which to draw the conclusion that there is "mit Ausnahme des Schweineblutes, ein auffallender Parallelismus zwischen der Höhe der Pyrophosphatfraktion einerseits, dem glykolytischen Vermögen der einzelnen Blutarten andererseits." The conclusions of these authors, however, find more support when their analytical data are compared with the extent of glycolysis in the blood of different animals as determined by Engelhardt and Ljubimowa (1930). Below is presented their arrangement of species in order of increasing glycolytic power, together with the average values found by Barrenscheen and Vasarhelyi (1930) for "pyrophosphate" in whole blood, and also our figures for the sum of nucleotide and hydrolyz-

<sup>5</sup> Glycolysis does not take place in pig blood, according to Engelhardt and Ljubimowa (1930).



able phosphorus (adenosine triphosphate) per 100 cc. of corpuscles for the same animals.

	Pig*	Ox	Goat	Dog	Horse	Guinea pig	Man	Rabbit
Barrenscheen and Vasarhelyi. Pyrophosphate in whole blood.....	7.83	1.48		2.77	0.97	4.98	5.85	5.42
Kerr and Daoud. Nucleotide P plus hydrolyzable P in corpuscles.....	33.6	5.4	5.9	10.9	4.5	12.2	18.3	23.7

\* See foot-note 5.

According to the data presented in the first series, the pig (with no glycolytic power), horse, and man appear to be exceptions to the conclusion of Barrenscheen and Vasarhelyi that glycolytic power is parallel to "pyrophosphate" concentration. However, our data differ from theirs in that we find rabbit blood to contain considerably *more* hydrolyzable phosphate than does human blood. This difference becomes more pronounced when the composition of the corpuscles rather than that of whole blood is examined. As for the horse, the position assigned to it in the series in accordance with its glycolytic power would fall between ox and goat if the one very divergent value in the four experiments of Engelhardt and Ljubimowa (1930) were to be discarded. From an examination of our data it appears therefore that (with the exception of the pig and possibly the horse) the glycolytic power of blood varies in the same direction as the sum of nucleotide and hydrolyzable phosphorus, which we interpret to represent adenosine triphosphate. Glycolysis is, of course, dependent upon a number of factors other than adenosine triphosphate. As Meyerhof (1932) has shown, the concentration of magnesium, the lactic acid-forming enzyme, and possibly also hexokinase play each a part in determining the rate and extent of glycolysis.

*Undetermined Phosphorus*—Since the quantities of nucleotide and hydrolyzable phosphorus are small compared to the total organic phosphorus in the blood of most species (oxen,<sup>6</sup> sheep,

<sup>6</sup> The value given by Lohmann (1928, *b*) for the organic acid-soluble phosphorus of ox blood (32.8 mg. of P per 100 cc.) is 10 times that of our

goats, and buffalo excepted), a large portion of the organic phosphorus remains undetermined in our experiments. A considerable portion of this is composed of diphosphoglyceric acid. Greenwald (1925) isolated from pig blood 19.1 mg. of P per 100 cc. (42.9 per cent of the total acid-soluble P) in the form of the barium salt of diphosphoglyceric acid, whereas in our experiments on pig blood the undetermined phosphorus averaged 58.2 per cent of the total. Hence, about 15 per cent remains undetermined in pig blood. From human and dog blood, however, Greenwald isolated only 20 to 22 per cent of the total phosphorus as diphosphoglyceric acid, while in our experiments 68 to 59 per cent remained undetermined. Further study of this fraction requires the development of methods for the determination of the diphosphoglyceric acid<sup>7</sup> and the two compounds Posternak (1928) claims to have isolated, *i.e.* the monophosphoric ester of *l*-glyceric acid and  $\alpha$ -ketotrihydroxyadipic diphosphate.

*Phosphocreatine*—Although phosphocreatine has been reported absent from blood (Martino, 1928; Eggleton and Eggleton, 1929), a number of investigators apparently believe it to be present (Schwarz and Taubenhaus, 1931; Bomskov, 1932; Geréb and Laszlo, 1932). The last named authors found higher values for inorganic phosphorus when determined by the direct colorimetric method (phosphocreatine plus inorganic) than when the inorganic phosphate was determined by precipitation with magnesia mixture, and interpreted this difference as evidence of the presence of phosphocreatine. In view of this uncertainty it is worth while to state that we have confirmed the results of Eggleton and Eggleton, and in three experiments found not a trace of phosphocreatine in the blood of dogs. Our procedure was to take the blood directly into 10 per cent trichloroacetic acid (iced), the amount of blood

determinations, while Barrenscheen and Vasarhelyi (1930) found approximately 5 times as much as we did. Our analyses for thirteen oxen (performed by three different persons) show the organic phosphorus of the *corpuses* to be lower than the values obtained by these workers for whole blood. Lohmann's figures for ox blood resemble ours for the pig. Our data agree with those of Kay (1928).

<sup>7</sup> The method used by Jost (1927) for determining diphosphoglyceric acid is based on the incorrect assumption that this substance is the only organic phosphorus compound which forms a water-insoluble lead salt.

added being determined by difference in weight. The filtrate was neutralized at once, treated with 0.25 volume of 10 per cent  $\text{CaCl}_2$  saturated with calcium hydroxide, and then filtered to remove the precipitated inorganic phosphate. Acid molybdate (Molybdate II of Fiske) was added, and after allowing half an hour for hydrolysis of phosphocreatine, the aminonaphtholsulfonic acid reagent was added. No visible blue color could be detected, indicating complete absence of phosphocreatine. As an additional check, a quantity of phosphate equal to that in the standard was added to the calcium filtrate, with the result that in the colorimeter standard and unknown were identical.

#### SUMMARY

1. The blood of twenty-four species of vertebrates was analyzed for organic acid-soluble phosphorus, purine nucleotide, and the fraction of organic phosphorus hydrolyzed in 7 minutes at  $100^\circ$  by normal acid. The concentration of these fractions in the erythrocytes was determined by calculation from cell volume.

2. In 76 per cent of the blood specimens the quantity of nucleotide was sufficient to account for all the "hydrolyzable" phosphorus as adenosine triphosphate. In relatively few of the remaining cases was the hydrolyzable phosphorus in sufficient excess of that calculated as adenosine triphosphate to indicate the presence of other easily hydrolyzed compounds of phosphorus. The sum of nucleotide and hydrolyzable phosphorus (which we interpret to represent adenosine triphosphate) ranges between 4 and 37 mg. of phosphorus per 100 cc. of corpuscles, representing 10 to 74 per cent of the organic acid-soluble phosphorus.

3. With the exception of pig and possibly horse blood, the glycolytic power of the blood of various species varies in the same direction as the content of adenosine triphosphate.

4. Phosphocreatine, reported present in blood by certain authors, we find to be absent from dog blood.

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# COMPLEX SALTS OF AMINO ACIDS AND PEPTIDES

## I. METAL COMPLEX SALTS OF GLYCINE AND THEIR SPECIFICITY

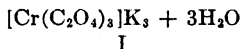
BY MAX BERGMANN AND SIDNEY W. FOX

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

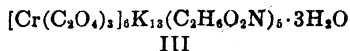
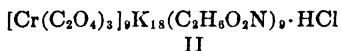
(Received for publication, January 21, 1935)

The mixtures of amino acids resulting from the hydrolysis of proteins and similar substances may be analyzed by the well known methods of Kossel and Kutscher, Fischer, Dakin, Van Slyke, Osborne, Vickery, and others. However, after hydrolyzing proteins with enzymes, one is faced with the difficult problem of determining the resultant amino acids and peptides without the occurrence of secondary splitting in the course of the analysis. To overcome this difficulty it is necessary to find stable metal complex salts capable of precipitating single amino acids or peptides. In this paper a reagent for glycine, to begin with the simplest amino acid, is described.

It was found that the long known and readily available potassium trioxalatochromiate (I) reacts with glycine.



It forms several well crystallized double salts containing both glycinium and potassium as cations. In the presence of hydrochloric acid in water-alcohol solution, the compounds (II) and (III) were found.



Compound (II), which contains glycine and chromium complex in equivalent amounts, results from solutions containing an excess of glycine. Without this excess, products usually result which have

the peculiar ratio of 5 equivalents of glycine to 6 chromium complex radicals as in (III).

Werner (1), studying in 1914 the reaction of glycine with potassium *trans*-dioxalatochromiate, obtained two compounds each of which, in contrast to ours, contained glycine inside the chromium complex. But the method of preparation and the general characteristics of these make them unsuitable for our purpose.

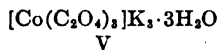
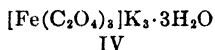
Pfeiffer (2) prepared a great number of addition compounds from amino acids and simple inorganic neutral salts. The glycine compounds described in the present paper are not addition compounds; they contain glycine as cation partly replacing the potassium ions of a neutral salt. Furthermore, Pfeiffer's salts were compounds of alkaline or alkaline earth halides with any of the following: glycine, sarcosine, alanine, glycylglycine, diglycylglycine, triglycylglycine, alanylglycine, betaines, and diketopiperazines.

We tried in vain, however, to place amino acids other than glycine in combination with potassium trioxalatochromiate.<sup>1</sup> Apparently, glycine is the only amino acid which forms a potassium trioxalatochromiate under the prevailing conditions. From hydrolysates of gelatin and keratin only glycine was precipitated as potassium trioxalatochromiate; other amino acids were not obtained in demonstrable quantities. This makes possible an estimation of glycine in hydrolysates of proteins and similar substances. The method which has been developed for this will be described in a later communication.

The selective behavior of potassium trioxalatochromiate towards glycine is unexpected. In complex chemistry generally, homologous substances give analogous compounds; *e.g.*, the fatty acids form a series of choleic acids; the amino acids form the numerous neutral salt compounds mentioned above. Phosphotungstic acid and Reinecke salt are metal complexes reacting with several different amino acids. If a mixture of amino acids is allowed to form salts with an acid, it is to be expected that most of the amino acids participate in salt formation. Here, however, glycine seems to be the only natural amino acid forming a salt and therefore one can speak of the specificity of potassium trioxalatochromiate for it.

<sup>1</sup> Even  $\alpha$ -aminoisobutyric acid, which resembles glycine in its symmetry, does not form a compound with salt (I).

The characteristics of the complex required for this specificity were next examined. It was found that the well known potassium trioxalatoferrate (IV) and potassium trioxalatocobaltate (V) also give a number of compounds with glycine, which are described in the experimental section. As regards constitution, they are analogous to the chromium compounds.



The iron derivatives with glycine are light, the cobalt dark green; both, like the greenish lilac chromium compounds, are dichroic. The cobalt compounds are characterized by their sensitivity to light and heat.

Glycine has been repeatedly separated from gelatin hydrolysates with the aid of these iron and cobalt salts. The selective affinity of the trioxalato metal complex towards glycine is therefore not altered by changing the central metal atom. The special nature of this metal atom is of secondary importance, for it acts only as the nucleus holding together the three oxalato groups. The outer region occupied here by these three oxalato groups determines the selective affinity for glycine.

It may be pointed out further that the nature of the cations, which are present in solution besides glycine, is important. The formation of a glycine-containing precipitate occurs only in the presence of other cations which can form with glycine and the trioxalato complex a sufficiently insoluble salt. In our experiments the potassium ion is capable of forming a glycine-containing oxalatochromiate. The ammonium may also; but not sodium. In treating glycine solutions with  $\text{Na}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 4\frac{1}{2}\text{H}_2\text{O}$ , all efforts to obtain a glycine-containing precipitate were negative. Even the glycine-containing precipitate obtained with the tripotassium salt is more or less redissolved after addition of sodium chloride. It does not suffice merely to have trioxalato metal, potassium, and glycine ions in solution to precipitate glycine; the other ions present may also affect the precipitation. There is a competition of several ions seeking equilibrium, which, under proper conditions, may lead to a specific precipitation of glycine.

In considering the specific precipitation of glycine, we may draw distinction between the specific reagent (trioxalato metal ion), the



supporting agents (*e.g.*,  $K^+$ ), and inhibiting agents (*e.g.*,  $Na^+$ ). The inhibitor functions by replacing the supporting agent and glycine in their combination with the specific reagent. Calcium and barium are more effective inhibitors than sodium. Barium forms with the tripotassium salt the compound  $[Cr(C_2O_4)_3]K_2Ba \cdot 2H_2O$  which is not inclined to exchange its potassium or barium for glycine. When glycine potassium trioxalatochromiate is treated with 1 molecular equivalent of barium chloride, there immediately results the nitrogen-free  $K_2Ba$  salt.

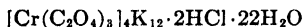
The distinction between specific, supporting, and inhibiting reagents exists in many biological specificity reactions. As examples there may be recalled the hemoglobins and enzymes which, according to Willstätter's theory, are composed of an active part and a so called carrier substance. It cannot be stated today whether or not similar phenomena occur in serological specificity reactions.<sup>2</sup>

We wish to acknowledge our indebtedness to Dr. A. Elek and Mr. J. L. Goldberg for making the analyses and to Dr. W. F. Ross for his assistance in preparing the manuscript.

#### EXPERIMENTAL

*Hydrochloride of Potassium Trioxalatochromiate*—If glycine or protein hydrolysates are treated in hydrochloric acid solution with tripotassium trioxalatochromiate, a nitrogen-free product is usually formed, which later changes into the glycine salt described below. The first product results from the action of  $HCl$  on the tripotassium salt.

2 gm. of tripotassium salt, when covered with 5 cc. of 2 N  $HCl$ , dissolved and soon the separation of well formed dichroic crystals began. The yield was increased by the addition of 5 cc. of alcohol. 1.8 gm. were obtained. The product was recrystallized by dissolving in 20 cc. of cold 2 N  $HCl$  and adding alcohol.



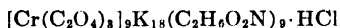
Calculated. C 13.1, H 2.1, Cl 3.2, K 21.3,  $H_2O$  18.0

Found. " 13.2, " 2.2, " 3.4, " 20.9, " 17.7  
" " " " " 18.2

<sup>2</sup> Cf. Landsteiner, K., *Die Specificität der serologischen Reactionen*, Berlin, 50-90 (1933).

*Salt of Glycine with Potassium Trioxalatochromiate. From Glycine*—The constitution of the resulting compound depends upon whether an excess of glycine is used.

7.5 gm. of glycine were dissolved in 60 cc. of 2 N HCl, treated with 10 gm. of tripotassium salt (I), filtered, and 60 cc. of alcohol added. The rosettes of flat prisms which formed were filtered off after standing a short while. Without being washed, they were dried on a porous plate.

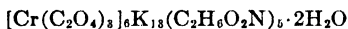


Calculated. C 20.2, H 1.4, N 3.0, K 16.5, Cl 0.8

Found. " 19.9, " 1.4, " 3.0, " 16.7, " 0.7

Although 5 molecules of glycine were present for each chrome complex, only one amino acid per chrome complex entered into the crystal. In the following experiments a 1:1 ratio of glycine to chrome complex was chosen; the proportion of amino acid in the crystal was, as a result, smaller.

1 gm. of glycine was dissolved in 25 cc. of 0.5 N HCl and 8 gm. of tripotassium salt were added. After shaking the flask for several minutes, 35 cc. of absolute alcohol were slowly added. The product consisting of microscopic needles was filtered after several hours. The yield was about 7 gm. It was recrystallized from 40 cc. of 0.1 N HCl by adding 50 cc. of absolute alcohol. The precipitation was controlled by observation under the microscope. Inasmuch as blue prisms appeared with the green needles of the glycine compound, the product was recrystallized for analysis. In this and the following cases, only products which appeared entirely homogeneous under the microscope were used for analysis.



Calculated. C 19.6, H 1.3, N 2.5, K 18.0, H<sub>2</sub>O 1.3

Found. " 19.7, " 1.4, " 2.5, " 17.9, " 1.1

The water content varies with the atmospheric humidity; salts with 3H<sub>2</sub>O and 4H<sub>2</sub>O were occasionally obtained. The preparations described contained at least 0.3 per cent Cl. Somewhat greater proportions were found when the precipitation and recrystallization of the glycine compound were conducted in an excess of N HCl.

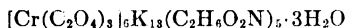
Found in crude product.

C 19.7, H 1.4, N 2.5, Cl 0.6

" " recrystallized product. " 19.8, " 1.5, " 2.5, " 0.5

*From Gelatin Hydrolysate*—5 gm. of gelatin (15 per cent H<sub>2</sub>O) were refluxed for 10 hours with 15 cc. of concentrated HCl. The solution was evaporated *in vacuo* and twice again treated with 10 cc. of H<sub>2</sub>O and evaporated. After being decolorized in water with charcoal, it was evaporated to 15 cc. 8 gm. of tripotassium salt and 15 cc. of 2 N HCl were now added, and after being shaken for 10 minutes 40 cc. of alcohol were added in portions. The dark N-free crystals, which first formed, soon changed into the green, microscopic needles of the glycine compound. After several hours the filtered crystals were washed with a solution of 1 part of concentrated HCl to 3 of alcohol, and dried on a porous plate; yield, 6.1 gm.

For analysis the material was twice dissolved in N HCl and precipitated with alcohol.



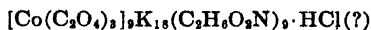
Calculated. C 19.5, H 1.3, N 2.5, K 17.9, H<sub>2</sub>O 1.9

Found. " 19.8, " 1.4, " 2.5, " 17.6, " 1.9

To confirm the presence of glycine, 5 gm. of the above trioxalatochromiate in aqueous solution were treated with 3 gm. of MgO and 2.8 gm. of benzoyl chloride. The precipitate formed with concentrated HCl was dried and washed with CCl<sub>4</sub>. 1.4 gm. (80 per cent of theory) were obtained. After recrystallization from water the melting point was 186–187° (uncorrected). Calculated, N 7.82; found, N 7.62.

*Salt of Glycine with Potassium Trioxalatocobaltiate*—There usually was formed a mixture of different glycine salts, and it proved difficult to isolate a homogeneous product. The crystallization often requires a day or more and in this time partial decomposition occurs even in the dark. Several procedures for preparing fairly homogeneous products are given.

*From Glycine*—7 gm. of tripotassium trioxalatocobaltiate, a great excess of glycine (5 gm.), and 38 cc. of 2 N HCl were shaken to effect as complete solution as possible. After being filtered the solution was treated with 50 cc. of alcohol, upon which dark green, flat prisms formed besides a very small quantity of needles. After 15 minutes they were filtered and dried on a porous plate in the dark. Yield, 7.2 gm.

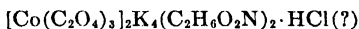


Calculated. C 19.8, H 1.3, N 2.9, K 16.2, Cl 0.8

Found. " 20.0, " 1.6, " 2.9, " 16.8, " 0.6

The analytical figures are not very exact because these complex salts may not be purified. On washing or recrystallization they may undergo great changes in composition. Therefore, this and several of the following formulas are given with reservations.

*From Gelatin Hydrolysate*—15 gm. of gelatin were hydrolyzed with HCl and, as above, brought to a volume of 35 cc. After being shaken for 10 minutes with 15 gm. of tripotassium trioxalatocobaltate, the solution was filtered off and the filtrate treated with 35 cc. of absolute alcohol. The resulting crystals changed rapidly into clusters of uniform, dark green prisms. After 45 minutes a small amount of colorless crystals, probably oxalate, appeared in the mass. To dissolve this, a mixture of 15 cc. of N HCl and 15 cc. of alcohol was added. The solid was then filtered off and dried in the dark on a porous plate. Yield, 7.2 gm.

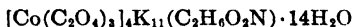


Calculated. C 19.4, H 1.6, N 2.8, Cl 3.7

Found. " 19.2, " 1.6, " 2.8, " 3.4

In order to prove the presence of glycine by transforming it into hippuric acid, it was necessary in this case to remove most of the cobalt complex as barium salt before benzylation. Hippuric acid was obtained in a yield of 85 per cent of the theoretical.

At another time the hydrolysate of 15 gm. of gelatin was treated with 6 gm. of tripotassium trioxalatocobaltate and gave a glycine compound quite different from the above. The product was recrystallized from 60 cc. of 0.5 N HCl by adding 60 cc. of absolute alcohol. The green needles formed were filtered immediately and dried in the dark at 6° on a porous plate. Yield, 1.85 gm.



Calculated. C 15.2, H 1.7, N 0.7, H<sub>2</sub>O 12.3

Found. " 15.3, " 1.6, " 0.7

Since the salt slowly decomposed on heating, the water content could not be determined accurately, but was estimated to be 12 to 13 per cent (calculated, 12.3 per cent).

*Salt of Glycine with Potassium Trioxalatoferrate. From Glycine*—A solution of 5 gm. of glycine and 7.5 gm. of tripotassium trioxalatoferrate in 40 cc. of 2 N HCl was filtered and treated with 40 cc. of absolute alcohol. The microscopic prisms which formed were removed after 15 minutes and dried on a porous plate in the dark. Yield, 7 gm.



Calculated. C 19.9, H 1.3, N 2.9, Cl 1.2, K 16.3, H<sub>2</sub>O 0.6

Found. " 19.8, " 1.5, " 3.1, " 1.1, " 16.8, " 0.6

Without an excess of glycine, a ratio of 5 equivalents of glycine to 6 metal complex radicals was found.

2 gm. of glycine and 15 gm. of tripotassium salt were dissolved in 50 cc. of 2 N HCl. 50 cc. of alcohol were added. On standing overnight six-sided plates, which overlay one another, were obtained. Yield, 14 gm.



Calculated. C 18.9, H 1.3, N 2.4, Cl 1.2

Found. " 18.5, " 1.5, " 2.4, " 1.2

*From Gelatin Hydrolysate*—The hydrolysate of 10 gm. of gelatin, brought to 20 cc. volume in the usual manner, was treated with 7 gm. of tripotassium trioxalatoferrate. The solution was warmed gently to dissolve all, filtered, and treated with 10 cc. of alcohol. After a few minutes the whole mass was a paste of prisms which were recrystallized from N HCl with alcohol. The resultant light green crystals were dried on a porous plate in the dark. Yield, 5 gm.



Calculated. N 2.9, K 15.9, Cl 3.6

Found. " 3.0, " 16.2, " 3.2

The glycine in this salt was converted into hippuric acid as with the cobalt salt. The yield was 75 per cent of the theoretical.

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## ON PROTEOLYTIC ENZYMES

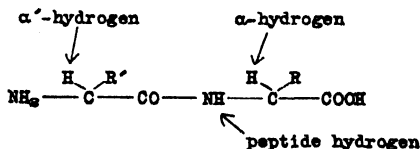
### V. ON THE SPECIFICITY OF DIPEPTIDASE\*

By MAX BERGMANN, LEONIDAS ZERVAS,† JOSEPH S. FRUTON,  
F. SCHNEIDER, AND H. SCHLEICH

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Dresden)

(Received for publication, January 18, 1935)

In recent years it has become possible to synthesize peptides of any desired amino acids and thus to obtain a wide selection of substrates for the investigation of the specificity of the proteolytic enzymes. It was found that dipeptidase splits all dipeptides corresponding to this general structure.



Thus it is essential for dipeptidase action that the substrate shall contain the peptide linkage, a free carboxyl, and an amino group. The carboxyl must be situated on the carbon atom next to the peptide nitrogen; the amino group must be on the carbon next to the peptide carbonyl. These results are in agreement with the conclusions drawn by Grassmann for the dipeptides of the simple amino acids (2). The peptide hydrogen is also essential (*cf.* Levene and Simms (3), Abderhalden *et al.* (4), and Bergmann *et al.* (5)). Finally, all dipeptides of natural amino acids have  $\alpha$ - and  $\alpha'$ -hydrogen in a definite spatial arrangement. If one of these 2 hydrogen atoms is missing, dipeptidase action is either completely or partly inhibited.

\* For Paper IV of this series see (1).

† Fellow of the Rockefeller Foundation.

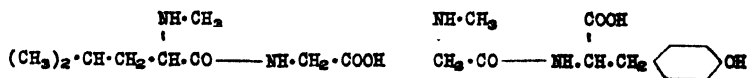
In the experiments here reported further information regarding these criteria of specificity has been obtained.

The essential character of the *amino group* had been drawn from the finding that N-acylated dipeptides were not split by dipeptidase. Upon acylation the basic character of the amino group is removed but is retained when an alkyl group is introduced instead of the acyl residue, as in sarcosylglycine which was first studied by Levene and his coworkers (6). We have examined the splitting of N-methyl-*dl*-leucylglycine (I), studied by Abderhalden and his coworkers (4, 7), because the methyl-free leucylglycine is very easily split and is therefore employed as a sensitive test substrate

TABLE I  
*Splitting with Glycerol Extract of Intestinal Mucosa*

Substrate	Time	Hydrolysis
	hrs.	per cent
<i>dl</i> -Leucylglycine	1	70
	2½	86
<i>dl</i> -Leucylglycylglycine	1	78
	2½	109
Chloroacetyl- <i>l</i> -tyrosine	2½	2
N-Methyl- <i>dl</i> -leucylglycine	2	4
	3½	2
Sarcosyl- <i>l</i> -tyrosine	2	1
	3½	2

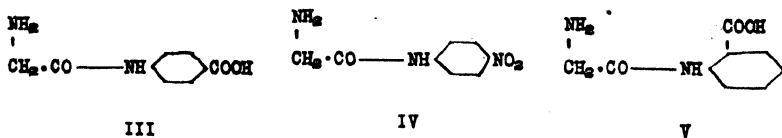
for dipeptidase. In agreement with Abderhalden's results, we could find no splitting with erepsin under the usual experimental conditions (Table I). A similar finding was obtained in the case of sarcosyl-*l*-tyrosine (II). Dipeptidase from intestinal mucosa requires, therefore, the complete  $\text{NH}_2$  group in the substrate and the basicity of the nitrogen is not in itself sufficient.



## II

The necessity of the *free carboxyl* in the peptide for dipeptidase action has been the subject of much research. Grassmann and his

coworkers (2) have demonstrated the essential character of the carboxyl group for the action of yeast dipeptidase. We have assumed the same for dipeptidase from intestinal mucosa and have never found in our experiments any evidence to the contrary. However, Balls and Köhler (8) have reported from the laboratory of Waldschmidt-Leitz that dipeptidase from intestinal mucosa splits dipeptides of aromatic amines. According to these investigators the enzyme is thus able to split glycyl-*p*-aminobenzoic acid (III) and glycyl-*p*-nitraniline (IV) but not glycyl-*o*-aminobenzoic



acid (V). None of these dipeptides bears a carboxyl group on the carbon atom attached to the peptide nitrogen. Peptide (IV) has no free carboxyl group at all.

Waldschmidt-Leitz and Balls (9) explain these results by attributing to the carboxyl group of glycyl-*o*-aminobenzoic acid an inhibiting effect on the enzyme. In the hydrolyzable *p* compound the carboxyl group is further removed, therefore the inhibition cannot occur. There can be no doubt that such inhibition by the carboxyl group is difficult to understand in an enzyme which, like dipeptidase, is capable of splitting compounds which have the peptide linkage and the carboxyl group in close spatial proximity. These results fit an enzyme which, like aminopeptidase, is inhibited by a free carboxyl in the vicinity of the peptide linkage of the substrate. It has been possible for us to demonstrate that aminopeptidase is indeed the active principle in this case.

Balls and Köhler employed for their observations a mixture of ereptic enzymes which contained, besides dipeptidase, much aminopeptidase. They did not perform experiments with homogeneous dipeptidase. We have repeated their experiments with yeast dipeptidase and have found that it is entirely inactive. On the other hand; it was possible to split glycyl-*p*-nitraniline as well as glycyl-*p*-aminobenzoic acid with aminopeptidase from intestinal mucosa (Tables II and III). It is therefore evident that if erepsin



TABLE II  
*Hydrolysis of Glycyl-p-Nitraniline*

Substrate	Glycerol extract of intestinal mucosa		Aminopeptidase		Yeast dipeptidase	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	hrs.	per cent	hrs.	per cent	hrs.	per cent
<i>dl</i> -Leucylglycine	1	100	1½	1	1	58
					3	100
<i>dl</i> -Leucylglycylglycine	1	80	1½	32	1	1
			3	77		
Glycyl- <i>p</i> -nitraniline*	2	9	3	6	2½	1
	5	24	7	7	5	0
	20	40	20	11	20	0

\* The amination of chloroacetyl-*p*-nitraniline (8) was carried out with ammoniacal methyl alcohol for 48 hours. After evaporating down the solution, the residue was dissolved in a measured volume of *N* HCl, filtered, and the peptide precipitated from the filtrate with the calculated quantity of *N* NaOH. Yellow needles, difficultly soluble in water, with a melting point of 167° (corrected), were obtained. The material was titrated in 0.3 cc. aliquots. During hydrolysis the color changed from light yellow (peptide) to deep yellow (nitraniline).

TABLE III  
*Hydrolysis of Glycyl-p-Aminobenzoic Acid*

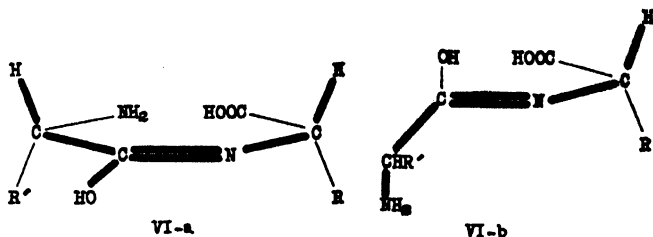
Substrate	Glycerol extract of intestinal mucosa*		Aminopeptidase		Yeast dipeptidase†	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	hrs.	per cent	hrs.	per cent	hrs.	per cent
<i>dl</i> -Leucylglycine	1	77	1½	1	2	83
<i>dl</i> -Leucylglycylglycine	1	80	1½	32	2	7
	2	102	3	77	7	38
Glycyl- <i>p</i> -aminobenzoic acid	2	19	3	7	3	0
	6	45	6	10	7	0
	15	69	20	20		

\* 0.3 cc. of extract was employed.

† 7 mg. of dry preparation were employed.

hydrolyzes these two dipeptides, as in the experiments of Balls and Köhler, the hydrolytic action is to be attributed to the aminopeptidase. Since the only experimental support for a difference in specificity for dipeptidase from yeast and intestinal mucosa has disappeared, it may now be stated without reservation that dipeptidase splits only those dipeptides which bear a free carboxyl group on the same carbon as the peptide nitrogen.

For the *stereochemical aspect of dipeptidase action* the peptide hydrogen, as well as the  $\alpha$ - and  $\alpha'$ -hydrogens, is of fundamental significance. It was pointed out above that the peptide hydrogen is essential for enzymatic hydrolysis by dipeptidase. We assume that this hydrogen atom becomes rearranged under the influence of the enzyme in such a way that the amide form of the dipeptide is changed to the imide form,  $\text{—CO—NH—} \rightarrow \text{—C(OH) = N—}$ . The imide form makes possible two different spatial arrangements of the carbon-nitrogen double bond.

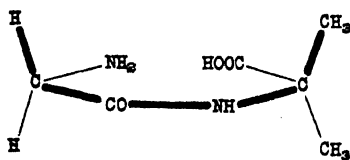


In isomer (VI-a) the amino and carboxyl groups lie in the closest possible proximity, whereas in (VI-b) they are distant from each other. We are of the opinion that during dipeptidase action dipeptides exist in the configuration (VI-a) so that there results a hexagon, whose corners are formed by the carboxyl group,  $\alpha$ -carbon, peptide nitrogen, carbon of the peptide bond,  $\alpha'$ -carbon, and amino group. The simultaneous combination of the carboxyl and the amino group with the enzyme restricts the free rotation within the peptide and confers upon the hexagon a rigid structure. The six corners of the hexagon must lie nearly in a single plane and we shall fall into no great error if we draw the hexagon as a plane and

refer to it as such in what follows. However, the 2  $\alpha$ -hydrogen atoms and the side chains R and R' are not situated in the plane of the hexagon but in a way similar to that of the substituents in a partially hydrogenated benzene ring.<sup>1</sup> The spatial configuration of the naturally occurring optically active amino acids is such that their  $\alpha$ -hydrogen atoms lie in *cis* position in the dipeptide hexagon, the same being the case for the side chains R and R'. On the one side of the hexagon plane there are therefore the two voluminous side chains R and R' which do not allow the enzyme to approach the hexagon plane from this side, thus preventing combination with the reactive groups of the dipeptide from this side. On the other side of the hexagon plane there are only the 2  $\alpha$ -hydrogen atoms which have only a small atomic volume. We are therefore of the opinion that the enzyme approaches that side of the hexagon upon which the 2  $\alpha$ -hydrogen atoms are situated and that this condition determines the stereochemical selectivity of dipeptidase.

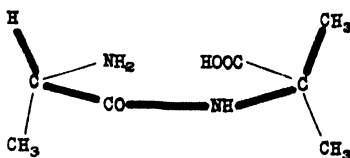
This view leads to the problem as to whether the 2  $\alpha$ -hydrogen atoms enter into active combination with dipeptidase or merely favor the action of the enzyme because of their small volume. The second possibility seemed the more probable one.

In order to decide this question we have investigated the following dipeptides with dipeptidase.



Glycyl aminoisobutyric acid

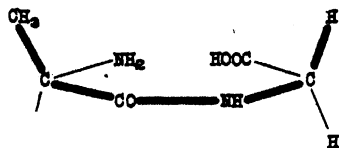
VII



L-Alanyl aminoisobutyric acid

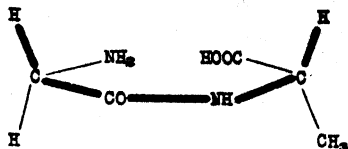
VIII

<sup>1</sup> In analogy to the Haworth formula of pyranosides, in formulas (VI-a) to (XVI) the heavy lines represent valences in front of the plane of the paper while the light lines represent valences behind the plane of the paper.



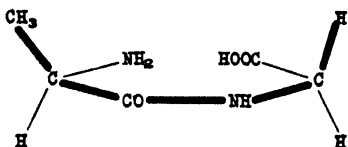
Aminoisobutyryl glycine

IX



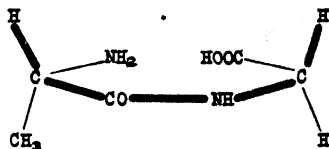
Glycyl-L-alanine

X\*



D-Alanyl glycine

XI



L-Alanyl glycine

XII

Abderhalden and Zeisset (10) found no splitting for dipeptide (VII) with erepsin but did observe hydrolysis in the case of (VIII) and of (IX), without stating which component of the erepsin was the effective agent in the splitting. We found hydrolysis by erepsin from intestinal mucosa for all three dipeptides. We found further that an active and homogeneous preparation of aminopeptidase from erepsin was entirely inactive toward these three dipeptides. Finally, we noted an appreciable splitting of all three dipeptides with a dry preparation of yeast dipeptidase. It was smallest in the case of (IX), where the aminoisobutyric acid bears the amino group (Tables IV to VI).

These experiments show that dipeptidase can also act on dipeptides of amino acids which have no  $\alpha$ -hydrogen but in its place a methyl group. The substitution of methyl for hydrogen on the side of the hexagon approached by the enzyme does not prevent the splitting by dipeptidase completely, but merely slows it down.

\* Since all natural amino acids have the same spatial configuration and belong to the so called *l* family, we designate them as *l* compounds and their antipodes as *d* compounds regardless of their optical behavior.

This is particularly clear when peptides (VII) and (IX) are investigated under the same conditions as glycyl-*l*-alanine (X) and *l*-alanylglycine (XII). The two last named peptides differ from (VII) and (IX) only in the fact that in place of the methyl groups on the upper side of the hexagon there are hydrogen atoms.

TABLE IV  
*Hydrolysis of Peptides of  $\alpha$ -Aminoisobutyric Acid*

Substrate	Glycerol extract of intestinal mucosa			Amino-peptidase		Yeast dipeptidase*	
	Time	Hydrolysis A	Hydrolysis B	Time	Hydrolysis	Time	Hydrolysis
	hrs.	per cent	per cent	hrs.	per cent	hrs.	per cent
<i>dl</i> -Leucylglycine	2	95	90	2½	3		
Glycylglycine						1	71
<i>dl</i> -Leucylglycylglycine	2	90	92	2½	96		
Chloroacetyl- <i>l</i> -tyrosine	1	1					
Glycylaminoisobutyric acid	5	5		3	3	3	4
	8		21	6	3	6	8
	8½	13				10	12
Glycyl- <i>l</i> -alanine	2		60				
	4		79				
<i>l</i> -Alanylaminoisobutyric acid	3½	62		3	0	1	16
	5	77		5½	0	3	35
	6½	89				10	45
Aminoisobutyrylglycine	3	22	24	3	0	3	25
	5	38		5	0	6	31
	6		51			10	38
	7	43					
<i>l</i> -Alanylglycine	3		65				
	6		91				

\* 4 mg. (0.4 peptidase unit) of dry preparation were employed per 1 cc. of reaction mixture.

At this point our experimental results and interpretation conflict with a specificity law whose general validity was held to be quite firmly established. It was stated that dipeptides containing only one antipode of a natural amino acid are not split by dipeptidase (11). Our proposed theory of the mechanism of dipeptidase

action requires, however, that certain exceptions to this rule should be observed. For example, *l*-leucyl-*d*-alanine (XIII) is composed of the natural *l*-leucine and the antipode of the natural

TABLE V

*Inhibition of Dipeptidase Action by Alanine. Glycerol Extract of Intestinal Mucosa Was Employed*

0.1 mm of *dl*-alanine was added to each reaction mixture.

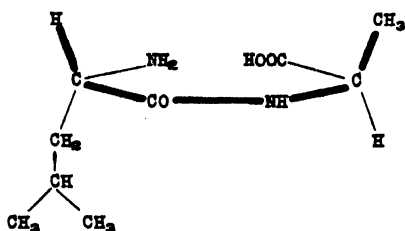
Substrate	Time	Hydrolysis	
		No alanine	Alanine
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>
Glycylglycine	1	30	18
	2	59	34
<i>l</i> -Alanylaminoisobutyric acid	2	36	24
	4	67	35
Aminoisobutyrylglycine	2	18	14
	4	29	16

TABLE VI

*Poisoning of Enzymatic Action of Extract of Intestinal Mucosa with HCN*  
3 mg. of HCN were added to each reaction mixture.

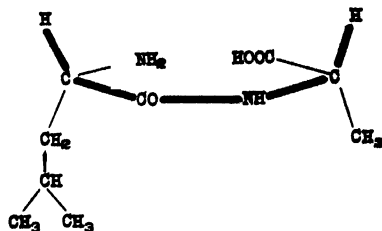
Substrate	Time	Hydrolysis	
		Without HCN	With HCN
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>
Glycylglycine	4	79	7
	6		7
<i>l</i> -Alanylaminoisobutyric acid	3		0
	5	77	0
Aminobutyrylglycine	3	22	0
	5	38	0

alanine. Its hexagon shows on its upper face precisely the same arrangement as in the two peptides (VII) and (VIII) of aminoisobutyric acid.



1-Leucyl-D-alanine

XIII



1-Leucyl-L-alanine

XIV

Since this is, however, the side of the hexagon upon which, according to our view, dipeptidase combines with the substrate, the enzyme should be able to combine with *l*-leucyl-*d*-alanine and thus split it. Similarly, *d*-alanylglycine (XI) should be hydrolyzed. Experiments with yeast dipeptidase have confirmed this expectation and shown further that *l*-leucyl-*d*-alanine is split more rapidly than the sluggish glycylglycine, but more slowly than *l*-leucylglycine or *l*-leucyl-*l*-alanine (XIV). Hydrolysis was also obtained in the case of *l*-leucyl-*d*-alanine with erepsin from intestinal mucosa, but not with aminopeptidase from the same source (Table VII).

It is of interest that aminoisobutyrylglycine is split more rapidly than *d*-alanylglycine. Thus the substitution of  $\alpha'$ -hydrogen by methyl on the side of the hexagon not approached by the enzyme in this case accelerates the splitting.

As a component of dipeptides subjected to dipeptidase action, *d*-alanine does not obey the hitherto accepted specificity rule. The rule must therefore be restricted as follows: If a dipeptide contains the antipode of a natural amino acid,  $\text{NH}_2 \cdot \text{CHR} \cdot \text{COOH}$ , and R is  $\text{CH}_3$  or larger, then steric hindrance of dipeptidase action results, which in the case of  $\text{R} = \text{C}_4\text{H}_9$  or bigger side chains<sup>2</sup> leads to complete inhibition of hydrolysis in the usual experimental period of time.

<sup>2</sup> The precise behavior of *d*-aminobutyric acid and the corresponding aminovaleric acid remains to be investigated. It was previously found (12) that the dipeptide, glycyldehydrophenylalanine, is not split by dipeptidase. In view of the results reported in this paper this finding cannot be explained by the absence of  $\alpha$ -hydrogen.

The following method for the determination of the configuration of primary amino acids was recently reported (13): combination with a natural amino acid to a dipeptide which is subjected to the action of dipeptidase. The validity of this method is not affected by the above remarks since it is employed only for amino acids which are much more complex than alanine.

TABLE VII  
*Hydrolysis of Peptides of d-Alanine*

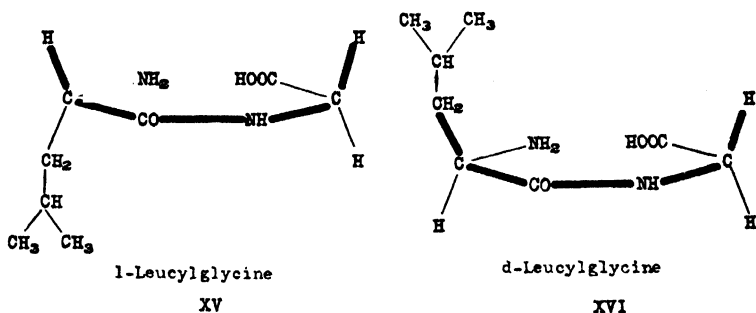
Substrate	Glycerol extract of intestinal mucosa		Aminopeptidase		Yeast dipeptidase*	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	hrs.	per cent	hrs.	per cent	hrs.	per cent
<i>dl</i> -Leucylglycine	1	70	2	0	2	74
	2½	86	4	0	4	90
Glycylglycine	1	39			2	36
	2½	74			4	80
					6	90
<i>dl</i> -Leucylglycylglycine	1	78	2	27	2	4
	2½	109	4	35	4	7
Chloroacetyl- <i>l</i> -tyrosine	2½	2				
Gelatin	2½	2				
<i>l</i> -Leucyl- <i>d</i> -alanine	1	26	2	0	2	25
	3	54	4	3	4	51
	5	70			6	69
<i>l</i> -Leucyl- <i>l</i> -alanine	1½	100				
<i>d</i> -Alanylglycine	1½	5			4	11
	4	13			8	28
	8	23				
	1½	42				
<i>l</i> -Alanylglycine	3	63				

\* 4 mg. of dry preparation were employed.

Since a large amount of consistent information regarding the specificity of dipeptidase is now at hand, it is worth while to discuss the cause for the stereochemical selectivity of the enzyme. The splitting of the peptides, glycylglycine, glycylaminoisobutyric acid, and aminoisobutyrylglycine, shows definitely that the enzyme requires no asymmetry of the amino acids or peptides. The fact



that stereochemical selectivity occurs in the case of peptides containing asymmetric amino acids will be elucidated with *l*-leucylglycine as an example. When the dipeptidase combines with *l*-leucylglycine on the upper side of the hexagon (*cf.* (XV)), it finds the carboxyl group, the peptide hydrogen, and the amino group arranged in a clockwise order. The reactive groups of the enzyme which combine with carboxyl, peptide hydrogen, and amino group must be arranged in the enzyme in such a manner that they fall exactly on the above groups in the peptide.



If we now subject *d*-leucylglycine (XVI) to dipeptidase action, we have again on the upper side of the hexagon the carboxyl group, peptide hydrogen, and the amino group arranged in clockwise order. However, the enzyme cannot combine with these groups because its approach is prevented by the voluminous  $C_4H_9$  group. Were the enzyme to act on the lower face of the hexagon, no splitting would result because the carboxyl group, peptide hydrogen, and amino group are arranged in a counter-clockwise order so that the reactive groups of the enzyme cannot combine with all the necessary reactive groups of the substrate.

If the enzyme would combine with only two reactive groups of the substrate, for example the amino group and the peptide hydrogen, it would not matter upon which side of the hexagon plane it reacts with the substrate and *d*-leucylglycine would be split exactly as its antipode. The last cause for optical selectivity of dipeptidase lies, therefore, in the fact that the enzyme combines with more than two points of the substrate. It seems probable that this theory of polyaffinity applies to other enzymes with asymmetrical specificity as well. We are investigating this question for car-

boxypeptidase and aminopeptidase and are using the steric hindrance of their action towards peptides of *d*-alanine as a convenient proof.

It may appear too early to propose such a detailed picture of the mechanism of dipeptidase action, especially in view of the lack of any knowledge regarding its chemical nature. However, the fact that the proposed theory could predict specificity phenomena (splitting of peptides of *d*-alanine) indicates that it possesses a high degree of probability.

#### EXPERIMENTAL

##### *Synthesis of Peptides of $\alpha$ -Aminoisobutyric Acid. Glycyl- $\alpha$ -Aminoisobutyric Acid*

*Carbobenzoxylglycyl- $\alpha$ -Aminoisobutyric Acid Ethyl Ester*—From 6.5 gm. of  $\alpha$ -aminoisobutyric acid ethyl ester hydrochloride a solution of the free ester in ethyl acetate was prepared in the usual manner. To this solution there were added with strong cooling 4 gm. of carbobenzoxylglycyl chloride (14) in several portions. After standing 1 hour at room temperature the  $\alpha$ -aminoisobutyric acid ester hydrochloride (2.3 gm.) which had separated out was filtered off by suction, the filtrate washed with dilute HCl, water, and bicarbonate, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated under diminished pressure. Needles with a melting point of  $84^\circ$  (corrected) were obtained. Yield, 4 gm.

For analysis the substance was recrystallized from ethyl acetate-petroleum ether.

$\text{C}_{16}\text{H}_{22}\text{O}_5\text{N}_2$  (322.2). Calculated, N 8.70; found, N 8.61

*Carbobenzoxylglycyl- $\alpha$ -Aminoisobutyric Acid*—4 gm. of the above ester were shaken for a short time with 14 cc. of  $\text{N NaOH}$  (1.1 moles) and 20 cc. of absolute alcohol, whereupon a clear solution was obtained. After 45 minutes the solution was acidified with dilute HCl, the free acid crystallizing out. On concentrating the filtrate under diminished pressure, a second crop of crystals was obtained. Total yield, 3.5 gm.; m.p.,  $154^\circ$  (corrected). The needles crystallized in clusters.

For analysis the material was recrystallized from water, whereupon the melting point rose to 155.5° (corrected).

$C_{14}H_{18}O_5N_2$ (294.2). Calculated, N 9.52; found, N 9.63

*Free Dipeptide*—1.4 gm. of the carbobenzoxy peptide were dissolved in absolute methyl alcohol, a small amount of water and 1 cc. of glacial acetic acid added, and the solution hydrogenated in the presence of palladium, Mohr, in the usual manner. The hydrogenation was completed in about 30 minutes. On evaporation under diminished pressure crystals (prisms) were obtained which were transferred to the filter by means of absolute alcohol. Yield, 0.7 gm.

For analysis the material was recrystallized from water-alcohol.

$C_6H_{12}O_4N_2$ .	Calculated.	C 44.98, H 7.55, N 17.49
160.1	Found.	" 45.26, " 7.46, " 17.48

3.360 mg. required 2.05 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.10 cc.

#### *l-Alanyl- $\alpha$ -Aminoisobutyric Acid*

*Carbobenzoxy-l-Alanyl- $\alpha$ -Aminoisobutyric Acid*—From 5 gm. of  $\alpha$ -aminoisobutyric acid ethyl ester hydrochloride a solution of the free ester in ethyl acetate was prepared in the usual manner. Into this solution there was filtered with cooling an absolute ethyl acetate solution of carbobenzoxy-*l*-alanyl chloride which was prepared (5) from 4.5 gm. of carbobenzoxy-*d*-alanine. After standing 3 hours the ester hydrochloride which had separated out was filtered off by suction, the filtrate washed with dilute HCl, water, and bicarbonate, dried over  $Na_2SO_4$ , and evaporated under reduced pressure. A syrup (3.8 gm.) resulted which could not be crystallized. This oil was dissolved in 20 cc. of absolute alcohol and shaken with 13 cc. of N NaOH for 1½ hours after cooling at the start. After acidifying with dilute HCl to Congo red acidity, most of the alcohol was removed by evaporation under reduced pressure. The aqueous residue was extracted with ethyl acetate; the ethyl acetate solution washed several times with water, dried over  $Na_2SO_4$ , and evaporated under reduced pressure. The crystalline residue was recrystallized from a small quantity of ethyl acetate. Yield, 2.2 gm. of lancet-shaped crystals.

For analysis the material was recrystallized from ethyl acetate. M.p., 165° (corrected).

$C_{15}H_{22}O_5N_2$  (308.2). Calculated, N 9.09; found, N 9.29

*Free Dipeptide*—1.7 gm. of the carbobenzoxy peptide were dissolved in absolute methanol, and after addition of a small quantity of water and 7 cc. of glacial acetic acid hydrogenated with palladium, Mohr, in the usual manner. The hydrogenation was completed in about 30 minutes. The filtered solution was evaporated under reduced pressure, giving in quantitative yield long prisms which were transferred to the filter by means of absolute alcohol.

For analysis the material was recrystallized from water and alcohol.

$C_7H_{14}O_3N_2$ .	Calculated.	C 48.28, H 8.10, N 16.09
174.1	Found.	" 48.61, " 8.15, " 16.17

4.125 mg. required 2.34 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.37 cc.  $[\alpha]_D^{20} = +34.52^\circ$  (2 per cent in  $H_2O$ ).

#### *$\alpha$ -Aminoisobutyrylglycine*

*Carbobenzoxy- $\alpha$ -Aminoisobutyric Acid*—From 14 gm. of  $\alpha$ -aminoisobutyric acid ethyl ester hydrochloride a solution of the free ester in ether was prepared in the usual manner. To this solution there were added with cooling 7 gm. of benzylcarbonyl chloride in several portions. After standing 4 hours at room temperature, the ester hydrochloride which had separated out (6.2 gm.) was filtered off by suction and the filtrate shaken with a little pyridine to decompose the unchanged chloride. The solution was washed with dilute HCl, water, and bicarbonate, dried over  $Na_2SO_4$ , and evaporated under diminished pressure. The resulting syrup (9.5 gm.) was dissolved in 25 cc. of absolute alcohol and shaken with 19 cc. of 2 N NaOH (1.1 moles) for 1½ hours with initial cooling. After being acidified with dilute HCl to Congo red acidity, most of the alcohol was removed by evaporation under reduced pressure. The aqueous residue was extracted with ether and the ethereal layer shaken with bicarbonate. The bicarbonate layer

was acidified with dilute HCl and extracted several times with ether. The ethereal solution was then washed with water, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated under diminished pressure, yielding a syrup which crystallized on standing and could be transferred to the filter by means of petroleum ether. Yield, 6.5 gm. of hexagonal plates.

For analysis the substance was recrystallized twice from ether-petroleum ether. M.p.,  $78^\circ$  (corrected).

$\text{C}_{13}\text{H}_{15}\text{O}_4\text{N}$  (237.1). Calculated, N 5.91; found, N 6.00

*Acid Chloride*—To a solution of 3 gm. of carbobenzoxy- $\alpha$ -aminoisobutyric acid in 20 cc. of absolute ether 3 gm. of  $\text{PCl}_5$  were added with strong cooling. After being shaken for 15 minutes, the filtered solution was evaporated under anhydrous conditions at  $0^\circ$  under diminished pressure, yielding a syrup which was washed several times with petroleum ether, and employed for the next reaction.

*$\alpha$ -Aminoisobutyrylglycine*—To a solution of about 4.5 gm. of free glycinebenzyl ester (15) in dry ethyl acetate was added a dry ethyl acetate solution of carbobenzoxy- $\alpha$ -aminoisobutyryl chloride (from 3 gm. of acid) described above. The filtrate of glycylbenzyl ester hydrochloride, which had separated out, was washed with dilute HCl, water, and bicarbonate, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated under diminished pressure. The resulting syrup was dissolved in absolute methanol; a few drops of water and 1 cc. of glacial acetic acid were added and hydrogenated with palladium, Mohr, in the usual manner. The hydrogenation was complete after  $1\frac{1}{2}$  hours. After the palladium was filtered off, the solution was evaporated under diminished pressure and after repeated evaporation with absolute alcohol yielded plates which were transferred to the filter by means of absolute alcohol. Yield, 0.5 gm.

For analysis the material was recrystallized from water-alcohol.

$\text{C}_8\text{H}_{12}\text{O}_3\text{N}_2$ .	Calculated.	C 44.98, H 7.55, N 7.49
160.1	Found.	" 45.28, " 7.44, " 7.38

3.805 mg. required 2.30 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.38 cc.

*l-Leucyl-d-Alanine*

*Carbobenzoxyl-l-Leucylhydrazide*—To a solution of 8.4 gm. of *l*-leucine methyl ester hydrochloride in 50 cc. of  $H_2O$  there were added 90 cc. of  $CHCl_3$  and then with strong cooling and shaking three 1 gm. portions of  $MgO$  and three 4 gm. portions of benzyl-carbonyl chloride were added. 15 minutes after the last addition the excess chloride was decomposed with pyridine and the reaction mixture acidified with 5 *N*  $HCl$ . The  $CHCl_3$  layer was washed twice with water, then with bicarbonate and dilute  $HCl$ , dried over  $Na_2SO_4$ , and evaporated under diminished pressure. A syrup resulted which was dried by repeated evaporation with methyl alcohol and dissolved in 50 cc. of absolute alcohol. 3.2 gm. of hydrazine hydrate were added and the reaction mixture was left at room temperature for 24 hours. A slight precipitate was filtered off; the alcohol evaporated off under diminished pressure and completely removed by two evaporations with ether. Needles; yield, 12.5 gm.; m.p.,  $121^\circ$ . For analysis the substance was recrystallized from ethyl acetate.

$C_{14}H_{21}O_5N_3$ .	Calculated.	C 60.21, H 7.88, N 14.69
279	Found.	" 59.99, " 7.76, " 14.54

*Carbobenzoxyl-l-Leucyl-d-Alanine Methyl Ester*—8 gm. of carbobenzoxyleucylhydrazide were dissolved in 100 cc. of  $H_2O$ , 10 cc. of acetic acid, and 5 cc. of concentrated  $HCl$ . With strong cooling ( $3^\circ$ ) and shaking, 3.5 gm. of  $NaNO_2$  were added slowly and the oil which separated out was extracted with ether. The ether solution was washed twice with cold  $H_2O$  and bicarbonate, filtered through a dry filter, and dried over  $Na_2SO_4$ . The dry ether solution of the azide was then mixed with a dry ether solution of *d*-alanine methyl ester from 3.2 gm. of *d*-alanine<sup>3</sup> and the reaction mixture was allowed to stand overnight at room temperature. Some of the substance crystallized out in long needles. These were filtered off, and the filtrate washed with dilute  $HCl$ , bicarbonate, and water,

<sup>3</sup> The *d*-alanine methyl ester was prepared from *d*-alanine hydrochloride which had been obtained by resolution of benzoyl-*dl*-alanine with brucine according to Fischer (16). The rotation of the *d*-alanine hydrochloride was  $[\alpha]_D^{25} = -10.2^\circ$  (10 per cent in  $H_2O$ ).

passed through a dry filter, and concentrated under diminished pressure. In this manner more of the substance was obtained. To the filtrate petroleum ether was added, yielding another crop. Yield, 3 gm.; m.p., 129–130°.

For analysis the substance was recrystallized from ethyl acetate and petroleum ether.

$C_{18}H_{26}N_2O_5$ .	Calculated.	C 61.71, H 7.45, N 8.00
350	Found.	" 61.63, " 7.46, " 8.00

This compound was also prepared by coupling carbobenzoxy-*l*-leucyl chloride with *d*-alanine methyl ester but the yield was even lower.

*Free Dipeptide*—A solution of 1.3 gm. of the ester in 7 cc. of acetone and 3.9 cc. of  $N$  NaOH (1.1 moles) was allowed to stand 40 minutes at room temperature with occasional shaking. The solution was filtered and to the filtrate 4.2 cc. of  $N$  HCl were added. The acetone was removed under diminished pressure, whereupon an oil appeared which could not be crystallized. It was extracted with ethyl acetate; the ethyl acetate solution dried over  $Na_2SO_4$  and evaporated under diminished pressure. The resulting syrup was dissolved in absolute methyl alcohol and hydrogenated with palladium as catalyst in the presence of acetic acid. The hydrogenation was completed in 30 minutes. The filtered solution was evaporated under reduced pressure and this procedure repeated twice after addition of absolute alcohol to remove all water and acetic acid. It was taken up in absolute alcohol and crystallized on standing in the ice box. Yield, 0.4 gm.

For analysis the substance was recrystallized from absolute alcohol.

$C_9H_{15}O_3N_2$ .	C 53.47, H 8.91, N 13.86
202	" 53.35, " 9.05, " 13.63

5.35 mg. required 2.69 cc. of 0.01  $N$  KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.65 cc.  $[\alpha]_D^{22} = +76.0^\circ$  (2.5 per cent in  $H_2O$ ).

#### *l*-Leucyl-*l*-Alanine

*Carbobenzoxy-l-Leucyl-l-Alanine Methyl Ester*—This was prepared in the same manner as in the case of *l*-leucyl-*d*-alanine. M.p., 92–93°.

$C_{11}H_{13}N_2O_5$ .	Calculated.	C 61.71, H 7.45, N 8.00
350	Found.	" 61.64, " 7.37, " 8.17

*Free Dipeptide*—This was prepared in the same manner as in the case of *l*-leucyl-*d*-alanine.

6.97 mg. required 3.45 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 3.39 cc.  $[\alpha]_D^{24} = +22.9^\circ$  (5 per cent in methyl alcohol).<sup>4</sup>

*d*-Alanylglycine

*Carbobenzoxyl-d-Alanine*—This was prepared in the same manner as the *l* form (14). M.p.,  $84^\circ$ .

$C_{11}H_{11}O_4N$ .	Calculated.	C 59.16, H 5.87, N 6.28
223	Found.	" 59.14, " 5.74, " 6.56

$[\alpha]_D = +13.9^\circ$  (8.5 per cent in glacial acetic acid).

*Carbobenzoxyl-d-Alanylglycine Ethyl Ester*—A dry ether solution of carbobenzoxyl-*d*-alanyl chloride prepared from 3.3 gm. of carbobenzoxyl-*d*-alanine in the same manner as the *l* form (5) was mixed with a dry ether solution of glycine ethyl ester prepared from 4.9 gm. of the hydrochloride. The reaction mixture was allowed to stand 30 minutes, whereupon some glycine ethyl ester hydrochloride separated out. This was filtered off and the filtrate washed with dilute HCl, bicarbonate, and water. The solution was then dried over  $Na_2SO_4$  and evaporated down under diminished pressure, whereupon the substance crystallized out in needles. Yield, 3.5 gm.; m.p.,  $98^\circ$ . For analysis the substance was recrystallized from ethyl acetate-petroleum ether.

$C_{16}H_{20}O_5N_2$ .	Calculated.	C 58.44, H 6.49, N 9.09
308	Found.	" 58.39, " 6.56, " 8.98

*Carbobenzoxyl-d-Alanylglycine*—To a solution of 2.5 gm. of ethyl ester in 8 cc. of acetone 10 cc. of N NaOH were added. After standing for 30 minutes with occasional shaking, the acetone was evaporated off, yielding an oil which crystallized on standing in the ice box. Yield, 1.7 gm.; m.p.,  $103-104^\circ$ . For analysis the substance was recrystallized from ethyl acetate.

<sup>4</sup> Fischer (17) gives  $[\alpha]_D^{20} = +23.5^\circ$ .



$C_{13}H_{16}O_5N_2$ .	Calculated.	C 55.71, H 5.71, N 10.00
280	Found.	" 55.61, " 5.67, " 10.01

*Free Dipeptide*—0.9 gm. of carbobenzoxy compound was dissolved in absolute methyl alcohol and hydrogenated in the usual manner after addition of a small amount of water and acetic acid. On evaporation under diminished pressure the peptide was obtained in a yield of 0.4 gm. For analysis it was recrystallized from  $H_2O$ -alcohol.

$C_{13}H_{16}O_5N_2$ .	Calculated.	C 41.08, H 6.90, N 19.18
146	Found.	" 41.01, " 6.95, " 19.23

7.30 mg. required 4.94 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 5.00 cc.  $[\alpha]_D^{23} = -50.7^\circ$  (4.3 per cent in  $H_2O$ ).<sup>5</sup>

#### *l-Alanylglycine*

*Carbobenzoxy-l-Alanylglycine Ethyl Ester*—This was prepared in the same manner as in the case of *d*-alanylglycine. M.p., 100°.

$C_{15}H_{20}O_5N_2$ .	Calculated.	C 58.44, H 6.49, N 9.09
308	Found.	" 58.41, " 6.44, " 9.16

*Carbobenzoxy-l-Alanylglycine*—This was prepared in the same manner as in the case of *d*-alanylglycine. M.p., 104°.

$C_{13}H_{16}O_5N_2$ .	Calculated.	C 55.71, H 5.71, N 10.00
280	Found.	" 55.64, " 5.75, " 10.21

*Free Dipeptide*—This was prepared by hydrogenation of the carbobenzoxy compound as in the case of *d*-alanylglycine.

7.17 mg. required 4.75 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 4.91 cc.

*Glycyl-l-Alanine*—This was prepared by the method of Fischer (19).

*N-Methyl-dl-Leucylglycine*—This was prepared by the method of Fischer (20).

*Sarcosyl-l-Tyrosine*—This was prepared by the method of Abderhalden, Schwab, and Valdecasas (4).

<sup>5</sup> Fischer (18) found for *l*-alanylglycine  $[\alpha]_D^{18} = +50.3^\circ$ .

The authors are grateful to Dr. A. Elek of New York and Miss J. Wetzel of Dresden for kindly performing the microanalyses reported in this paper.

### *Enzymatic Studies*

In the experiments reported above the total volume of each reaction mixture was 1 cc. in which were contained 0.05 mM of substrate<sup>6</sup> in 0.5 cc. of H<sub>2</sub>O previously neutralized with 0.2 N NH<sub>4</sub>OH, 0.1 cc. of M/3 phosphate buffer of the required pH (usually 7.4), and 0.4 cc. of the enzyme solution. The temperature in all cases was 40°.

The extent of hydrolysis was determined by titration of the carboxyl groups in 90 per cent alcohol with 0.01 N KOH, thymolphthalein being used as indicator (Grassmann and Heyde (21)). 100 per cent splitting represents an increase of 1 cc. in the titration of 0.2 cc. of the reaction mixture.

The aminopeptidase employed in these experiments was prepared from a glycerol extract of intestinal mucosa by adsorption of the dipeptidase with Fe(OH)<sub>3</sub> according to Waldschmidt-Leitz, Balls, and Graser (22).

The dry preparation of yeast dipeptidase was prepared according to Grassmann and Klenk (23).

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<sup>6</sup> In the case of *dl*-peptides 0.1 mM was employed and splitting calculated only for the natural form.

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## THE INDISPENSABILITY OF ZINC IN THE NUTRITION OF THE RAT\*

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In a previous paper from this laboratory Todd, Elvehjem, and Hart (1) presented data demonstrating that zinc was essential in the nutrition of the rat. They found that the rate of growth of rats on a synthetic diet low in zinc was accelerated by the addition of salts of this metal. There was also an interference with the development of a normal fur coat of the animals on the zinc-low diet. Shortly before the publication of this work, Newell and McCollum (2) reported that no consistent differences in growth could be detected in animals receiving a ration in which there was almost a complete absence of zinc (their analysis showed a concentration of the order of  $10^{-7}$  or less) and control animals on the same ration to which zinc had been added. They did find, however, the zinc content of the rat body to be variable and somewhat dependent upon the zinc content of the diet; also that the young born to a female which was on a zinc-low diet had a greatly diminished zinc content as compared with young born to a female on a stock diet. They concluded that zinc was probably not an essential nutritional factor in the growth of the rat.

As a result of these conflicting data we considered it desirable to continue our work on this problem. More recently Bertrand and Bhattacharjee (3) showed that mice placed on a synthetic diet containing less than 0.5 mg. of zinc per kilo died in 14 to 23 days. The litter mates which received 20 parts per million of zinc and served as controls lived for 57 to 74 days.

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The basal ration which we have used, though supplemented with concentrates of all the known vitamins and zinc, did not produce normal growth of the animals. While the zinc content of the ration was below the minimal requirements, the animals receiving added zinc did not respond maximally because their growth was retarded by an insufficient supply of certain other unknown factors. Our problem, therefore, was to supply these factors in the ration so that the animals would grow normally and thus obtain a clear cut picture of the zinc deficiency. We had found that by supplementing our ration with 2 cc. of milk per day per animal growth was markedly accelerated. Because this quantity of milk supplied an appreciable amount of zinc, it was necessary to prepare from milk or other food materials a fraction low in zinc which would add these growth factors. The original basal ration was also modified slightly in an attempt to reduce the zinc intake of the animals. Since Newell and McCollum reported that they were able to remove zinc from casein by electrodialysis, we attempted to use this method for the purification of whole milk and whey. This method of purification, however, proved ineffective, and it became necessary to rely upon precipitation methods for the preparation of the water-soluble growth factors from milk.

#### EXPERIMENTAL

*Animals*—All the animals used in the experiments were specially handled to reduce the zinc stores as much as possible. The pregnant females were placed in the monel metal cages several days before parturition. They were fed the stock colony ration, milk, and water *ad libitum*. When the young were 15 days old, the stock colony ration was replaced by the zinc-low ration and redistilled water. From this time until the young were weaned the mothers were removed from the young for 2 hours twice daily to receive the stock ration, milk, and water. In this way the young never obtained the stock ration and gradually became accustomed to eating the zinc-low ration, so that by the 25th day, when they were weaned and started on the experiment, there was no abrupt change in their diet. Each litter was divided into two groups, one group on the zinc-low ration, and the other on the same ration with added zinc.

*Ration*—The source and procedure for the preparation of the

carbohydrates, protein, and salts were the same as those reported in our former paper (1).

*Fat*—Pure butter fat in which was dissolved sufficient haliver oil to supplement it with ample amounts of the fat-soluble vitamins replaced Wesson oil which was formerly used. The butter was purified by placing  $1\frac{1}{2}$  pounds of unsalted butter in a liter beaker and allowing it to melt in an oven at  $80^{\circ}$ . The melted oil was stirred to permit the curd and water to settle to the bottom, after which the clear oil was decanted off. 1 gm. of haliver oil, which contained 30,000 units of vitamin A (United States Pharmacopoeia X) and 3333 units of vitamin D (Steenbock), was dissolved in 800 gm. of the butter oil.

Each gm. of the resulting fat contained 37.5 units of vitamin A and 4.2 units of vitamin D. This product was stored in the ice box and each day 0.4 gm. was measured into individual dishes. Each animal received in this amount of fat 15 units of vitamin A and 1.7 units of vitamin D in addition to what was present in the butter. The zinc content of this preparation was exceedingly low; 1 kilo contained 0.36 mg. of zinc and the daily ingestion of zinc from this source amounted to only 0.14 microgram.

*Water-Soluble Factors*—An attempt was made to remove the zinc from whole milk by electrodialysis. The apparatus used was a two compartment cell consisting of an outer 14 liter glass battery jar in which was suspended a porous clay cylinder with a capacity of 1.5 liters. A platinum coil served as the anode in the center compartment. The cathode was a piece of copper gauze which surrounded the porous cylinder. The material to be dialyzed was placed in the center compartment and a stream of purified air forced through the solution kept it well stirred and cool. The current was supplied by a d.c. generator and with external resistances the current was not permitted to exceed 1.5 amperes. After 2 hours the internal resistance of the cell increased so that with a potential of 137 volts only 0.6 ampere passed through the cell. The pH of the substance dialyzed would drop as low as 3 when samples were dialyzed for 20 to 40 hours. The temperature in the outer chamber was kept below  $40^{\circ}$  by changing the distilled water every 2 hours.

The whole cow's milk was obtained by milking directly into a glass container to eliminate contamination. Previous analysis of

such milk showed the zinc content to vary from 2.7 to 3.2 mg. per liter with samples taken from different cows at different times. Since the milk after it was subjected to dialysis for 18 to 43 hours still contained 2.2 to 3.0 mg. of zinc per liter, only a small amount, if any, was removed by this process. Whey dialyzed for periods as long as 26 hours still contained from 2 to 2.3 mg. per liter. Two samples of whey which had been previously concentrated 4:1 *in vacuo* below 25° and then dialyzed showed a zinc content of 1.5 mg. per liter when the calculations were made on the reconstructed basis. An attempt was also made to prepare casein in the same manner as that outlined by Newell and McCollum (2). Skim milk was dialyzed for 2 hours. By this time the casein was coagulated and floated on the surface. It was filtered and washed with redistilled water. Two samples of casein prepared in this manner had a zinc content of 55 mg. per kilo on the dry basis. Casein prepared by our method contained only from 3 to 6 mg. of zinc per kilo (1).

The results obtained proved electrodialysis to be entirely unsatisfactory for the removal of zinc from these products. This is not in accordance with the work of Newell and McCollum, who report casein prepared in this way to be spectrographically free from zinc. Their casein alone undoubtedly introduced enough zinc to supply amounts adequate to meet the requirements of the growing animal, therefore no differences could be detected in the control animals which received this ration with additional zinc.

Owing to the failure of electrodialysis in the removal of zinc from these milk products, a procedure for the preparation of a zinc-low milk serum was developed. 6 gallons of skim milk were placed in a porcelain jar, the temperature brought to 40°, and 10 cc. of rennet added. After 10 minutes the curd was carefully cut with a steel knife. The temperature was slowly raised to 46°, thereby producing a tough curd which settled to the bottom within 4 hours. The resulting whey, which was clear and free from casein particles, was drawn off and allowed to stand at room temperature for 24 hours; this permitted the lactic acid organisms to lower the pH to the isoelectric point of the albumin (pH 4.5). It was then placed in a 2 liter Erlenmeyer flask and heated in a boiling water bath with frequent stirring until the temperature reached 88°, which required about 15 minutes. The hot material was then

placed in the ice box overnight to cool and permit the albumin to settle. The clear serum was siphoned off and concentrated ammonium hydroxide added until a flocculent precipitation of the phosphates occurred (pH 9). This precipitate, which contained the zinc, settled to the bottom. The supernatant liquid was withdrawn, and by the addition of acetic acid the pH was lowered to 6. This serum was stored in the ice box and a new preparation made every 3 weeks.

Assays of the serum for vitamin B<sub>1</sub> were made in the same way as those reported in our previous paper. When 15 cc. were administered daily to animals which had ceased to grow on the vitamin B<sub>1</sub>-deficient diet, they made gains of 40 to 50 gm. in the 4 weeks following. The zinc content of the serum ranged from 0.2 to 0.25 mg. per liter. The daily ingestion of 15 cc. introduced 3.7 micrograms of zinc. The milk serum contained all the salts found in milk except the calcium phosphates which were removed along with the zinc in the ammonium hydroxide precipitation. For this reason the salts in the basal ration were reduced from 4 per cent to 3.5 per cent. Since casein introduced considerable zinc, it was reduced from 14 to 5 per cent and the egg white, which was practically zinc-free, was increased from 5 to 14 per cent.

The basal zinc-low ration used in our experiments contained the following ingredients.

	<i>per cent</i>
Casein.....	5
Egg white.....	14
Starch.....	77.5
Salts 45-T (1).....	3.5
15 cc. milk serum per animal daily	
0.4 gm. butter fat per animal daily*	
Redistilled water <i>ad libitum</i>	

\* In Experiment I Wesson oil reinforced with haliver oil replaced the butter fat.

The control ration was prepared by adding 6 mg. of zinc as ZnCl<sub>2</sub> per 100 gm. of this ration.

The basal ration was fed *ad libitum* in glazed porcelain dishes. Scattering of the ration was prevented by inverting a Petri dish containing a small opening over the top of the dish. This feeder proved very satisfactory in preventing wastage of ration. The



serum and butter fat were fed together in small porcelain dishes. Both products were readily consumed by all animals.

The growth curves for the five animals in Experiment I are shown in Chart I. At the end of the 4th week the two animals on the ration with added zinc had gained twice as much as the controls on the zinc-low ration.

After the 8th week the animals receiving the ration with added zinc failed to continue to grow normally. (Rat 14 ♂ remained

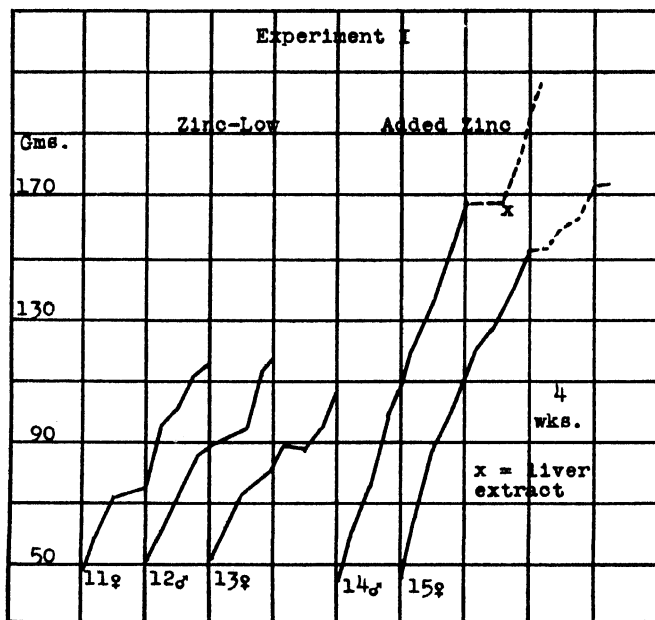


CHART I. Effect of adequate zinc intake on growth of rats

at 165 gm. for 2 weeks.) An alcoholic extract of liver equivalent to 4 gm. of fresh liver was administered daily and this animal gained 40 gm. in the 3 weeks following; the control animal, which did not receive the extract, gained only 11 gm. in this time.

As a result of this observation, liver extract was used to supplement the milk serum in all the three following experiments and beneficial effects were noted. For its preparation 900 gm. of fresh ground liver were extracted three times with 800 cc. of 95 per cent

alcohol. The total extract was combined and after cooling on ice for several hours the precipitated proteins were centrifuged off and the fluorescent liquid concentrated under a vacuum and below 40° to 30 cc. 500 cc. of 95 per cent alcohol were added and the precipitated proteins centrifuged off. The liquid was then concentrated to 30 cc. The last step was repeated once more. When assayed for vitamin B<sub>1</sub>, an amount of the extract equivalent to 4 gm. of the fresh liver produced a favorable growth response, but this was not equal to that produced by 15 cc. of milk serum. When the two were fed together the growth response was not greater than when the serum was fed alone, therefore indicating that its supplementary action was not due to a deficiency of vitamin B<sub>1</sub> in the 15 cc. of serum. 20 cc. of serum alone also produced no greater growth response than 15 cc. No zinc could be detected in an amount of extract equivalent to 200 gm. of the fresh liver. The extract equivalent to 4 gm. of liver therefore contained not over 1 microgram of zinc.

During the late winter months difficulties were encountered in the preparation of the zinc-low milk serum owing to some unknown change in the milk system. This change occurred during the last week in January of 1934 and persisted until the latter part of May when the cows were again back on pasture. This year (1934) milk changed to the winter condition in the latter part of October (1934) or about 3 months earlier than in the preceding winter. The difficulty encountered in the preparation of the serum after the milk had undergone this change to the winter condition was that the phosphate precipitate obtained by the addition of ammonium hydroxide to the serum could not be easily separated, since it remained in a colloidal state.

Because of this difficulty in the preparation of the serum, the animals in Experiment IV, which was started on May 4, 1934, were given liver extract as the sole source of the water-soluble factors for the first 3 weeks of the experiment. It was then again possible to prepare the serum so that thereafter both the liver extract and serum were supplied. The basal ration for Experiments IV, V, and VI was the same as that used in Experiment I. The only changes were the addition of liver extract to supplement the serum and the replacement of Wesson oil by butter fat.

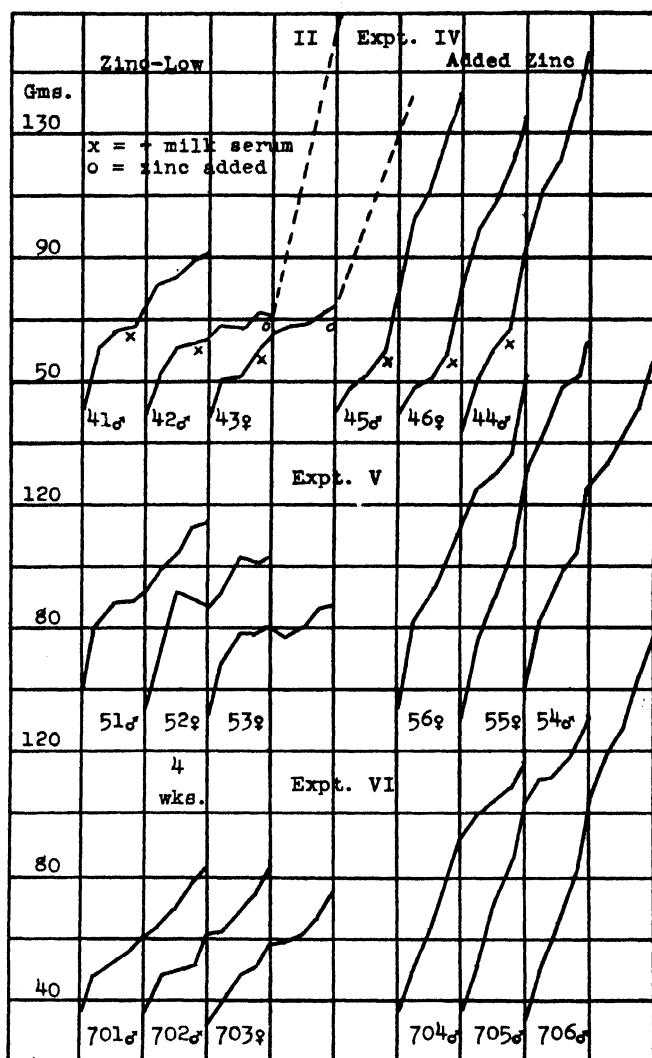


CHART II. Effects of zinc on growth of rats

*Results*

The growth curves for each of the four experiments indicate clearly that the growth of the animals on the zinc-low ration was markedly retarded. Chart II shows the growth records of the animals in Experiments IV, V, and VI. In several cases the gains made by animals receiving added zinc were 3 times as great as those made by the controls in the 8 week period. No specific pathological disturbances were detected in the animals on the zinc-low ration. They were, however, extremely active and hyper-irritable, and if removed from the cage would run about as if

TABLE I  
*Food Consumption Records*

Experiment No.	Average daily food intake		Ratio $\frac{\text{ration}}{\text{gain}}$	
	Zn-low	Added Zn	Zn-low	Added Zn
	gm.	gm.	gm.	gm.
IV	3.6	4.9	3.8	2.6
V	4.4	5.6	4.4	2.7
VI	4.0	5.2	4.3	2.8
Average, IV, V, VI.	4.0	5.2	4.1	2.7

The average daily food intake of the nine zinc-low animals for the 8 week period was 4 gm. The average consumption for the nine control animals was 5.2 gm. per day over the same period. In addition to this each animal received 0.4 gm. of butter, 15 cc. of serum, and liver extract.

hungry and in search of food. After 6 to 7 weeks the fur became soft and woolly and lost its luster. The black portions of the fur became light gray in color. The control animals were apparently normal in every respect and did not exhibit these nervous symptoms. Their fur remained normal throughout the experiment.

Food consumption records were kept for all animals on these experiments. The records for Experiments IV, V, and VI show that the animals on the ration with added zinc consumed 30 per cent more than those receiving the zinc-low ration during the 8 week period. The food consumption of the males and females was of the same order, making it possible to obtain an accurate average of the daily consumption of the animals in each group. These data are shown in Table I.

When the two groups are compared with respect to the efficiency of conversion of gm. of ration consumed into gm. of increase of body weight, the zinc-low animals required 52 per cent more ration to make a gm. of gain in weight. The food consumption of the animals on the ration containing added zinc was not limited to that of the basal groups because the object was to compare the effects produced in the one case by the deficiency of zinc and in the other the effects produced when this deficiency was eliminated by its addition. Since the animals receiving zinc were more efficient converters of the ration into body weight, they most probably would have made more rapid gains than those on the basal diet if the amount of food consumed by the animals in each group had been the same.

It is possible to calculate the daily zinc intake of the animals. 20 kilos of the zinc-low ration showed a zinc content of 2.2 mg. per kilo. The animals on the zinc-low ration consumed less than 4.5 gm. of the basal ration daily. Their daily ingestion of zinc amounted to less than 15 micrograms.

	<i>micrograms</i>
4.5 gm. basal ration.....	9.9
15 cc. serum.....	2.7
Liver extract.....	1.0
Butter fat.....	0.15
	<hr/> 14.75

Zinc analyses were made on the entire carcasses of the five animals of Experiment I. The total zinc per carcass of the three animals on the zinc-low ration ranged from 2.8 to 3.7 mg. or 21 to 26 mg. per kilo of live weight. The amount of food consumed by these animals up to the time they were killed for analysis introduced not over 1.1 mg. of zinc per animal. Therefore the rest of the zinc (1.7 to 2.6 mg.) was the amount present in the animals at the start of the experiment plus that obtained from other sources. From the amount of ration consumed by the two animals which had received added zinc each obtained 28 mg. of zinc. Analysis showed the carcasses of these animals to contain 12 and 17 mg. or 70 to 85 mg. of zinc per kilo of body weight. The entire carcasses of three stock males were also analyzed and found to contain from 59 to 63 mg. of zinc per kilo of live weight.

The per cent of dry matter in all the animals was nearly the

same. The average per cent of dry matter in the zinc-low animals was 36.6 as compared to 37 for the two animals which received added zinc. Thus the added zinc produced body tissue with no increase in water content.

At the end of the 8 week period, two animals, Rats 42 ♂ and 43 ♀ of Experiment IV, were placed on the ration with added zinc and they resumed normal growth as shown in Chart II. In 4 weeks the thin woolly hair coat was restored to normal and the gray portions became black, the same as in the control animals which had received zinc throughout the experimental period.

#### DISCUSSION

The results presented in this paper further substantiate our former work which demonstrated that zinc was an essential element in the nutrition of the rat. The basal ration has been improved by increasing its growth-promoting properties through the addition of certain water-soluble factors from milk serum, and at the same time reducing its zinc content. As a result of these improvements greater differences in the rates of growth of the animals on the zinc-low and zinc-containing rations have been obtained.

Our results are in accordance with those reported by Bertrand and Bhattacharjee (3). Their animals on the basal ration plus zinc lived only 74 days while our rats have been on the experiment for 14 weeks and appear normal in every way. Therefore our ration is more complete in other essential elements although it contains slightly more zinc than their rations.

The only explanation which we can offer of the contradictory results reported by Newell and McCollum (2) is that their rats were receiving an adequate supply of zinc. It seems probable that the casein in their ration supplied some zinc, since we have been unable to obtain zinc-low casein according to the procedure described by them. Furthermore, we have been unable to prepare a vitamin concentrate from yeast, according to their method, which was low in zinc.

Although no experiments have been conducted with other animals, it is logical to assume that if zinc is an essential element in the case of rats and mice, it is also needed by all animals. The difficulties which have been encountered in preparing a zinc-low

ration suggest that most natural rations contain a sufficient amount of this element. However, it is significant to note that a naturally occurring zinc deficiency in plants has been reported by Finch and Kinnison (4) as a result of their studies on pecan rosette. This disease of the pecan tree, which occurs in certain areas of Arizona, is promptly alleviated by administration of zinc salts. Analysis of the drainage waters from areas where this disease occurs showed a very low zinc content. In areas such as these zinc may be a limiting factor in both plant and animal nutrition. At present the function of zinc in the living organism is still unknown. In this connection attention might be called to the work of Maxwell (5) who found that the presence of small amounts of zinc salts in hypophyseal extracts produced a marked augmentation in the ovarian weight increase of young female rats. He also found the augmentation with urine of pregnancy preparations to be increased when zinc salts were added to the hypophyseal synergistic component.

Scott (6) reported that insulin contained 1.46 per cent ash and gave positive tests for iron, zinc, and phosphorus. By subjecting the insulin to electrodialysis the ash content was reduced to 0.04 per cent and tests for iron and phosphorus were negative. A positive test for zinc was always obtained, indicating that the zinc was firmly bound in the insulin molecule.

#### SUMMARY

1. Further evidence has been presented showing that the growth of rats on a synthetic diet low in zinc was markedly retarded as compared to control animals which received the same diet with added zinc.

2. On the zinc-low diet there was interference with the development of a normal fur coat.

3. The evidence presented demonstrates that zinc is an essential element for the growth of the rat. How it functions is still unknown.

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# A NOTE ON THE TITRATION CONSTANTS OF IMIDAZOLE DERIVATIVES

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(Received for publication, January 22, 1935)

In considering the behavior of histidine in the formol titration, it became necessary to have some information about the titration constants of imidazole derivatives. The constant of histidine near pH 2 is typical of a carboxyl group in amino acids and that near 9.2 is typical of an amino group. The other constant, near 6, by elimination, must belong to the imidazole group (2, 5, 11). Although the writer had no doubts of the validity of this assignment, it seemed desirable to determine the constants of other imidazole derivatives for comparison and particularly to learn what effect the presence of other groups might have on the imidazole constants. The substances studied were 4-(or 5-)methylimidazole, imidazolelactic acid, histamine, and histidine.

Data are available in the literature for histidine (2, 5, 9) and its peptides (6). Richardson (8) titrated *dl*-2-thiolhistidine but instead of the four titration groups expected found only three. He believes that the inaccessible group is the imidazole nitrogen.

## EXPERIMENTAL

Electrometric titrations were carried out on 0.01 M solutions at 30°. The hydrogen electrode in conjunction with a saturated calomel half-cell was used. The system was standardized, - 0.0647 volt being used as the potential of the hydrogen electrode in 0.1 M HCl. The analysis of the titration data presented no special difficulties. The titration constants were calculated on the basis of the Bronsted (3) definitions and apply to the standard Henderson-Hasselbalch equation for an acid.

We prepared 4-(or 5-)methylimidazole through the zinc salt

(1) and oxalate (7). The oxalate was converted to the free base according to Windaus and Knoop (12). Its purity was checked by the melting point ( $56^{\circ}$ ). Imidazolelactic acid was prepared by the action of  $\text{AgNO}_2$  on histidine monohydrochloride (4). Histamine dihydrochloride and histidine monohydrochloride were commercial products of excellent grade. All the substances showed the theoretical acid equivalent.

### Results

The titration constants are given in Table I. Consideration of the constants confirms the usual allocation in histidine. The position of the constant of the imidazole group is markedly influenced by the presence of an  $\text{NH}_3^+$  group  $\beta$  to it. Thus compari-

TABLE I  
*Titration Constants of Imidazole Derivatives at  $30^{\circ}$*

Substance	Group		
	Carboxyl	Imidazole	Amino
4-(or 5-)Methylimidazole.....		pG = 7.40	
Imidazolelactic acid.....	pG <sub>1</sub> = 2.99	pG <sub>2</sub> = 7.32	
Histamine.....		pG <sub>1</sub> = 5.90	pG <sub>2</sub> = 9.70
Histidine .....	pG <sub>1</sub> = 1.65	pG <sub>2</sub> = 6.01	pG <sub>3</sub> = 9.17
" (calculated)*.....	" = 1.81	" = 5.93	" = 9.07

\* From the data of Schmidt, Kirk, and Appleman (10).

son of histamine with methylimidazole and with histidine shows that the presence of the amino group shifts the constant to a region about 1.4 units more acid. Comparison of methylimidazole and imidazolelactic acid shows that the presence of the carboxyl group has very little influence on the position of the imidazole constant. The allocation of the constants is given in Table I. The values in the last line were calculated from the constants at  $25^{\circ}$  and the heats of ionization given by Schmidt, Kirk, and Appleman (10).

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# EQUILIBRIA OF THE BASIC AMINO ACIDS IN THE FORMOL TITRATION

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(Received for publication, January 22, 1935).

In a previous paper (5), the behavior of a number of amino acids in the formol titration was systematized by the use of hypothetical equilibria and the appropriate constants. The present communication deals with an extension of this system to the basic amino acids—arginine, lysine, and histidine.

The titration curves of these amino acids may be treated as equivalent to the curves obtained by titrating mixtures of three monovalent acids<sup>1</sup> present in equivalent quantities and having the requisite titration constants (8, 9). Each of the single acids behaves in the mixture according to the Henderson-Hasselbalch equation and the amino acids can therefore be characterized by three constants,  $pK_1$ ,  $pK_2$ , and  $pK_3$ .

Birch and Harris (1) have published titration curves, at one or two formaldehyde concentrations, for each of the basic amino acids. These curves are of the standard form predicted by the Henderson-Hasselbalch equation. This fact has been confirmed in the course of the present work. The problem of the behavior of the basic amino acids in the formol titration is, therefore, to determine the laws governing the changes of the titration constants with formaldehyde concentration. The data of Birch and Harris are too few to give an adequate basis for the solution of the problem.

## EXPERIMENTAL

The experiments were conducted at 30° in the manner previously described (5, 6), suitably modified to fit special peculiarities. To

<sup>1</sup> The term acid is used in the Bronsted sense.

investigate the variation of a particular group, a solution was prepared by dissolving the desired substance in water and adding the requisite quantity of NaOH or HCl to bring it to the mid-point ( $\text{pH} = \text{pK}$ ) of the appropriate group. The resultant solution was diluted to volume and titrated with formaldehyde, the hydrogen electrode being used to measure the pH after each addition. The amino acid concentration was 0.01 M within 20 per cent, the variation being due to dilution by the formaldehyde solution. Arginine did not come to equilibrium rapidly enough for this method. It was necessary to prepare a series of solutions at different formaldehyde concentrations and to measure the pH after 24 hours. The pH did not then change in a further 48 hours. The course of the pH changes was investigated and found to follow smooth curves, which will be discussed in a later section.

The materials used and their amino acid contents, determined by the Van Slyke method, are given below.

	Amino N	
	Found	Calculated
	<i>per cent</i>	<i>per cent</i>
Arginine monohydrochloride.....	6.68	6.64
Lysine dihydrochloride.....	12.79	12.79
Histidine monohydrochloride (hydrate).....	6.67	6.68
Histamine dihydrochloride.....	7.48	7.60

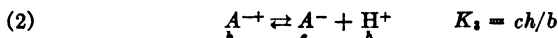
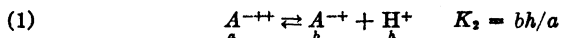
In every case the titration values checked the expected within 1 or 2 per cent. No other data than the titration value are at hand on the imidazolelactic acid used because of the small quantity available.

### Results

The experimental results are presented in the form of graphs showing the relations between the mid-point pH ( $\text{pG}_f$ ) values and the logarithms of the formaldehyde concentrations (see Figs. 1, 2, and 4). As records of the experimental results, the graphs seem self-explanatory. Detailed discussion will be found with the development of working equations and the calculations of constants for the postulated equilibria.

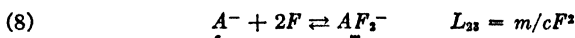
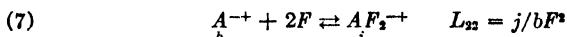
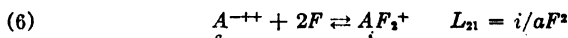
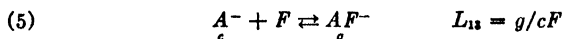
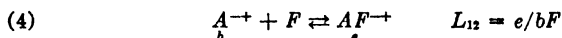
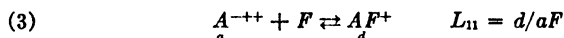
*Theoretical*

In order to unify the treatment a hypothetical amino acid conforming to the type under discussion will be considered. At the isoelectric point it is symbolized by  $A^\pm$ . Its behavior in an acid-base system, aside from the most acid group (which is not involved in the formol titration) may be represented by Equations 1 and 2.



The lower case letter below each symbol is used to represent both the concentration and the species in the equations and discussion. The  $K$  values are the hydrogen ion dissociation constants as determined in water.

The entities represented by  $a$ ,  $b$ , and  $c$  may be capable of reacting with 1 or 2 molecules of formaldehyde as shown in the following reactions. The  $L$  values are the formaldehyde association constants and are identified by systematic subscripts.



In the equations the lower case letters are used as before and the capital  $F$  represents the formaldehyde and its concentration.

The above equations were not, of course, written *a priori*, but are used to serve as a convenient framework to show the basic similarity of the varied experimental curves. Not all the equilibria are necessary to describe the data in the specific case of each amino acid. Some of the particular reactions do not occur at all or they occur in inaccessible pH regions or formaldehyde concen-



trations. In Table I are listed the formulæ of the compounds of arginine, lysine, and histidine corresponding to the generalized type of acid given above. A space without a formula indicates that for one of the reasons just stated equilibria involving the entity omitted need not be considered in deriving working equations. Study of Table I will show that formaldehyde is considered as associating with the  $\text{NH}_2$  group only.

Besides the appropriate set of reactions among those above the analysis of the curves involves the principle that the sums of positive and negative ions shall be equal. In most cases the  $\text{H}^+$  and  $\text{OH}^-$  ions do not contribute significantly to these sums, so that they are usually omitted from the equations for electroneutrality.

The development of working equations and the determination of the constants are discussed separately for each amino acid.

The multiplicity of the constants might make it appear that they are of little practical value in the actual use of the formol titration. For some of the constants this is certainly true. For practical purposes a single combination constant for each amino acid is more convenient. In the discussion of stoichiometry such constants are used. These with the other constants are collected in Table II under the heading  $\text{pG}_F$ . This is defined as the apparent titration constant of the most alkaline group titrated in the formol titration in 2.3 M formaldehyde. This is the constant which determines the end-point to be used with the conditions previously recommended (6).

### *Arginine*

The region in which the acidity of the guanidine group of arginine becomes manifest is also a region in which the buffering power of formaldehyde is very great. The preponderance of the necessary corrections makes an accurate estimate of how much  $\text{pK}_3$  is affected by formaldehyde very uncertain from the data available.<sup>2</sup>

<sup>2</sup> The implication in a recent note by Jukes and Branch (Jukes, T. H., and Branch, G. E. K., *Science*, **80**, 228 (1935)) that the lack of a demonstrable titer for the guanidine group in the formol titration proves that it does not react with formaldehyde is incorrect. The apparent strength of this group may have been increased by formol, without affecting the titer, to pH 9 if the constant of the group concerned still remained above 11.

When formaldehyde is added to arginine in a solution at  $pK_2$  (pH 8.91), there is, as stated above, a change of pH with time. This change was investigated for a number of formaldehyde concentrations and a family of curves obtained. The rate of the change increases with the concentration. It is probable that the electrode did not follow the changes as rapidly as they occurred. An attempt was made to derive kinetic equations which would fit, but the results were not consistent. It is enough to note that the major part of the change is soon over (5 minutes) even in  $m$   $CH_2O$  and that it is not dependent on the presence of the electrode or of  $H_2$ . The points in Fig. 1 were obtained after 24 hours standing and did not change in a further 2 days. The lower formaldehyde concentrations have been corrected for the amount required to react with arginine.

The points in Fig. 1 can be described in terms of Equations 1, 6, and 7 combined with the equation for electroneutrality  $a + i = b + j$ . The resultant equation is Equation 9.

$$(9) \quad pG_f = pK_2 - \log(1 + L_{22}F^2) + \log(1 + L_{21}F^2)$$

When  $L_{22}F^2$  is large compared to 1 and  $L_{21}F^2$  small

$$(10) \quad pG_f = pK_2 L_{22} - 2 \log F$$

Under these conditions the curve in Fig. 1 is a straight line with a slope of 2. The situation is realized over the lower part of the curve and the extrapolation of this part of the curve to  $\log F = 0$  as indicated by the dotted line gives  $pK_2 L_{22} = 3.50$ .  $pL_{22} = -5.41^3$  or  $L_{22} = 2.57 \times 10^5$ . This is a much larger association constant than any observed for the monoamino acids.

When  $F$  is large, Equation 9 reduces to a constant  $pG_f = pK_2 L_{22}/L_{21}$ . The constant value which  $pG_f$  approaches when  $F$  is large is 3.35.  $pL_{21}$  is therefore 0.15.

To show the agreement of the equations with the data the curve of Fig. 1 is plotted from the constants and the points are experimental.

It is interesting to note that  $pK_2 L_{22}/L_{21}$  is the hydrogen ion dissociation constant of the formaldehyde compound of arginine indicated by  $i$  in Table I. Its value, 3.35, is typical of a carboxyl

<sup>3</sup> The symbol  $p$  is used as an operator with the usual significance as in pH, etc.

**TABLE I**  
*Correlation of Generalized Amino Acid with Arginine, Histidine, and Lysine*

Type	Symbol	Formulae		
		Arginine G, guanidine N	Lysine X, 1 or 2 molecules of formaldehyde	Histidine I, imidazole N
$A^{-++}$	$a$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_{11}\text{N}_2-\text{NH}_3^+ \\ \diagdown \quad \diagup \\ \text{GH}^+ \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_3^+ \\ \diagdown \quad \diagup \\ \text{NH}_3^+ \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_6\text{N}-\text{IH}^+ \\ \diagdown \quad \diagup \\ \text{NH}_3^+ \end{array}$
$A^{-+}$	$b$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_{11}\text{N}_2-\text{NH}_2 \\ \diagdown \quad \diagup \\ \text{GH}^+ \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_2 \\ \diagdown \quad \diagup \\ \text{NH}_3^+ \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_6\text{N}-\text{I} \\ \diagdown \quad \diagup \\ \text{NH}_3^+ \end{array}$
$A^{-}$	$c$		$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_2\text{X} \\ \diagdown \quad \diagup \\ \text{NH}_2 \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_6\text{N}-\text{I} \\ \diagdown \quad \diagup \\ \text{NH}_2 \end{array}$
$AF^+$	$d$			$\begin{array}{c} \text{COOH} \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_6\text{N}-\text{IH}^+ \\ \diagdown \quad \diagup \\ \text{NH}_2(\text{CH}_2\text{O}) \end{array}$
$AF^{-+}$	$e$		$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_2(\text{CH}_2\text{O}) \\ \diagdown \quad \diagup \\ \text{NH}_3^+ \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_6\text{N}-\text{IH}^+ \\ \diagdown \quad \diagup \\ \text{NH}_2(\text{CH}_2\text{O}) \end{array}$
$AF^{-}$	$g$		$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_2\text{X} \\ \diagdown \quad \diagup \\ \text{NH}_2(\text{CH}_2\text{O}) \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_6\text{N}-\text{I} \\ \diagdown \quad \diagup \\ \text{NH}_2(\text{CH}_2\text{O}) \end{array}$
$AF_2^+$	$i$	$\begin{array}{c} \text{COOH} \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_{11}\text{N}_2-\text{NH}_2(\text{CH}_2\text{O})_2 \\ \diagdown \quad \diagup \\ \text{GH}^+ \end{array}$		
$AF_2^{\pm}$	$j$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_{11}\text{N}_2-\text{NH}_2(\text{CH}_2\text{O}) \\ \diagdown \quad \diagup \\ \text{GH}^+ \end{array}$	$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_2(\text{CH}_2\text{O})_2 \\ \diagdown \quad \diagup \\ \text{NH}_3^+ \end{array}$	
$AF_2^{-}$	$m$		$\begin{array}{c} \text{COO}^- \\ \diagup \quad \diagdown \\ \text{C}_6\text{H}_9-\text{NH}_2\text{X} \\ \diagdown \quad \diagup \\ \text{NH}_2(\text{CH}_2\text{O})_2 \end{array}$	

group in a compound carrying a distant positive charge and is therefore reasonable for this substance.

*Stoichiometry*—Sørensen (10) found that arginine monohydrochloride required 1 equivalent of alkali in the formol titration and that this was practically independent of the formaldehyde concentration and the pH of the end-point. This conforms to the high value of  $L_{22}$ . With the conditions recently recommended, arginine

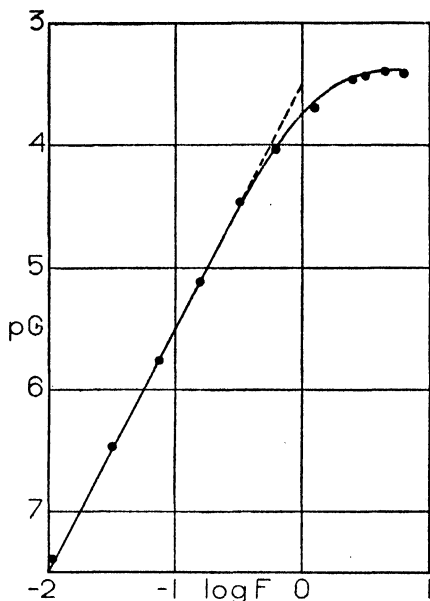


FIG. 1. The titration constants of arginine in formaldehyde. The ordinate represents  $pG$ , starting at  $pK_2$ ; abscissa, logarithms of the formaldehyde concentrations in moles per liter.

should be titrated completely plus a small blank. If sufficient time is allowed to obtain equilibrium, quite low formaldehyde concentrations would allow complete titration to an end-point of 7 and with pure arginine these conditions would be much more favorable than the usual formol titration. In mixtures, however, a titration of this kind would also include a considerable amount of histidine and a lesser amount of lysine. Monoamino acids would probably not interfere.

*Lysine*

The titration constants associated with the two amino groups of lysine are changed by formaldehyde. Because these groups overlap somewhat, the stoichiometric mid pH values are not exactly equal to the pK values of the groups concerned. Formaldehyde produces nearly parallel changes in the titration constants of the two groups and the discrepancy between mid pH and pK is

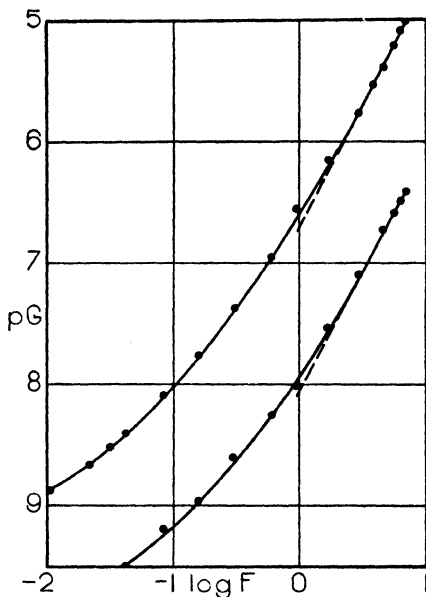


FIG. 2. The titration constants of lysine in formaldehyde. The coordinates are the same as in Fig. 1. The lower curve is started at  $pK_1$  and the upper at  $pK_2$ .

small in any case. The interpretation of the data is considerably simplified if the difference is ignored and the stoichiometric mid pH treated as equal to pK or  $pG_f$  as the case may require.

In Fig. 2  $pG_f$  values are plotted against  $\log F$ . The curves are of the same general form as those obtained with the monoamino acids (5). The same form of equation describes them.

When the curve starts with  $pK_2$ , the equations are developed

from Equations 1, 4, and 7 and the statement of electroneutrality  $a = b + e + j$ . The manipulations described in (5) lead to

$$(11) \quad \left( \frac{G_f}{K_2} - 1 \right) \frac{1}{F} = L_{12} + L_{22}F = 100M$$

In Fig. 3  $M$  is plotted against  $F$ . The slope of the straight line is  $0.01 L_{22}$  and the intercept  $0.01 L_{12}$ . Another way of getting the constant  $L_{22}$  is to take advantage of the simplified equation

$$(12) \quad pG_f = pK_2 L_{22} - 2 \log F$$

which indicates that the part of the upper curve in Fig. 2 having a slope of 2, when extrapolated to  $\log F = 0$ , should have the intercept  $pK_2 L_{22}$ . The values of the constants are given in Table II.

TABLE II  
*Constants Determining Behavior of Basic Amino Acids in Formaldehyde*

Amino acid	pK <sub>2</sub>	pK <sub>3</sub>	pL <sub>11</sub>	pL <sub>12</sub>	pL <sub>13</sub>	pL <sub>21</sub>	pL <sub>22</sub>	pL <sub>23</sub>	pG <sub>F</sub>
Arginine.....	8.91					0.15	-5.41		3.45
Lysine.....	9.11	10.56		-1.95	-2.38		-2.40	-2.49	7.35
Histidine.....	6.00	9.17	0.23	-2.62	-4.5				7.90
	pK <sub>1</sub>	pK <sub>2</sub>							
Histamine.....	5.95	9.70		-2.59	-4.5	-2.86			

In considering the variation of the second amino, pK<sub>3</sub>, group of lysine account must be taken of the reactions used in developing pG<sub>f</sub>. Would the association of formaldehyde with  $b$  affect the tendency of the second amino group to dissociate hydrogen ion? The reaction of formaldehyde with  $b$  does not change the electrical charge produced by ionization. The charges seem to be the chief influences affecting the dissociation tendencies of other groups in the molecule. The answer to the question is therefore most probably negative. The test is to use the constant pK<sub>3</sub> determined in the absence of formaldehyde in the equations developed. This was done and found to fit satisfactorily. This justifies the writing of 1 or 2 molecules of formaldehyde associated with the first amino group in  $c$ ,  $g$ , and  $m$  as indicated by  $X$  in Table I. The hydrogen ion dissociation tendencies of  $b$ ,  $e$ , and  $j$  are equal.

The equation for electroneutrality at the mid pH of the second amino group is  $b = c + g + m$ . Equations 13 and 14 are analogous to Equations 11 and 12.

$$(13) \quad \left( \frac{G_f}{K_3} - 1 \right) \frac{1}{F} = L_{13} + L_{23}F = 100M$$

$$(14) \quad \text{p}G_{f_2} = \text{p}K_3L_{23} - 2 \log F$$

The plot of Equation 13 on Fig. 3 gives a straight line with intercept  $L_{13}$  and slope  $L_{23}$ . The extrapolation of the lower curve in

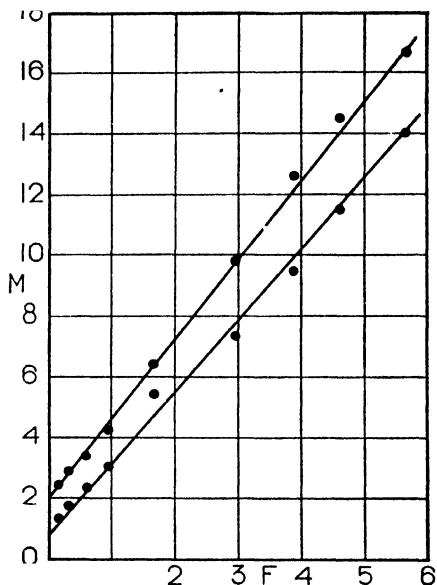


FIG. 3. The formaldehyde association constants of lysine. The ordinate represents  $M$  of Equations 11 (lower curve) and 13 (upper curve); abscissa,  $F$  in moles per liter.

Fig. 2 in the proper manner gives  $\text{p}K_3L_{23}$ . The values of the constants may be found in Table II.

*Stoichiometry*—The amount of alkali used in the titration of lysine in the formol titration depends on the initial pH as well as on the end-point (11). At the usual initial pH, 7, lysine is present as the monocation. Complete titration therefore requires that 2 equivalents of alkali be used. The pH of the end-point is deter-

mined by  $pG_{\gamma}$ . With the conditions and equations developed in a previous paper (6) and  $pK_3L_{23} = 8.1$ , the stoichiometric point is  $pH_{s'} = 9.7 + 0.5 \log C$  where  $C$  is the lysine concentration at the end-volume. But in a mixture an end-point 0.1 unit more acid than this was recommended. By taking this into account the error in the titration of the last group would be  $0.42/\sqrt{C}$ . At 0.1 M lysine this would be 1.3 per cent. The other group would be completely titrated and the total error would be 0.65 per cent. In dealing with pure lysine the more favorable calculated end-point would give an error half as great.

Sørensen investigated the stoichiometry of lysine and, with conditions under which other amino acids were nearly completely titrated, found that 92.5 per cent of the theoretical for one group was required. He started from the isoelectric point so that one group should have been titrated. Jodidi (3) explained this "abnormality" by assuming that the feeble basicity of one amino group affected adversely its ability to combine with formaldehyde. Actually the association constants of lysine are between 5 and 10 times as great as those for most monoamino acids. The greater these constants are the more favorable the acid is for accurate titration. Responsibility for the low values obtained would more justly be placed on the high value of  $pK_3$ . Sørensen's error is greater than that calculated above because he corrected for a blank which is not allowed for in the equations (6).

### *Histidine*

Kossel and Edlbacher (4) adjusted an imidazole solution to pH 7, added formaldehyde, and titrated with alkali to a thymolphthalein end-point. The quantity of alkali required was 84 to 85 per cent of the theory for one group. If the  $pK$  of imidazole is like that of its methyl derivative (7), this result would have been obtained whether the formol had been added or not. The lack of a control without formol led these workers to state that their experiment demonstrated a reaction between the imidazole and formaldehyde similar to that of amino groups. The experiment does not do this.

In order to determine whether or not the imidazole group reacts with formaldehyde, experiments were performed with 4-(or 5-) methylimidazole and with imidazolelactic acid. The solutions



were adjusted to the mid-points of the imidazole groups by the addition of the requisite amount of acid or alkali and titrated with formaldehyde. The results are recorded in Curves I and II of Fig. 4. The effect is very much less than with an amino group. This may be taken to indicate that the imidazole group does not associate formaldehyde (the effect is then ascribed to solvent changes) or that both the acid and corresponding base react to only slightly different extents.

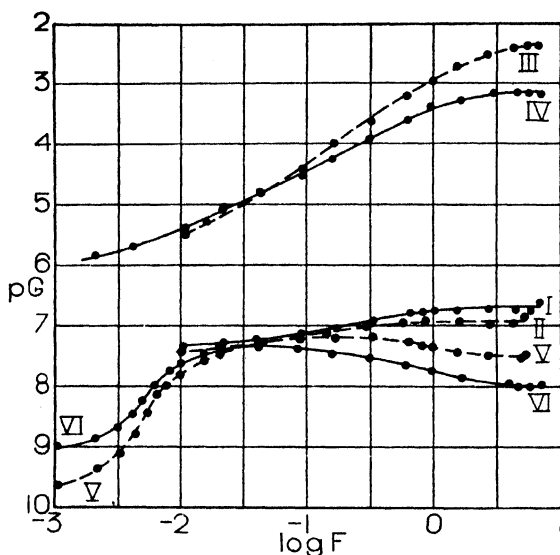


FIG. 4. The titration constants of imidazole derivatives in formaldehyde. The coordinates are the same as in Fig. 1. Curve I, 4-(or 5)-methylimidazole; Curve II, imidazolelactic acid; Curve III, histamine starting from  $pK_1$ ; Curve IV, histidine, starting at  $pK_2$ ; Curve V, histamine starting at  $pK_2$ ; Curve VI, histidine starting at  $pK_1$ .

In the case of histidine the major changes produced in  $pG$  by formaldehyde are interpretable on the basis that the imidazole group does not react and the formulæ have been so written in Table I.

The data for Curve IV in Fig. 4 were obtained on the addition of formaldehyde to a histidine solution originally at pH 6, the mid-point of the imidazole group (7). The equilibria which must be

considered are represented in Equations 1, 3, and 4. The equation for electroneutrality is  $a + d = b + e$ . At low formaldehyde concentrations  $d$  is small and may be neglected. Then Equation 15 may be derived.

$$(15) \quad h = K_2 + K_2 L_{12} F$$

This equation is plotted in Fig. 5. From the slope of the line  $K_2 L_{12}$  is  $4.10 \times 10^{-4}$  or  $pL_{12} = -2.62$ .

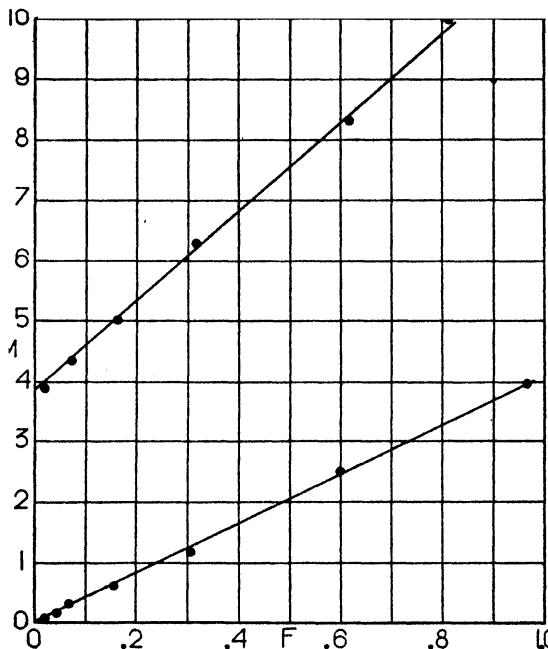


FIG. 5. The formaldehyde association constants of histidine and histamine. Abscissa,  $F$  in moles per liter; ordinates: upper curve,  $M$  of Equation 16, lower curve,  $H \times 10^4$  (Equation 15).

When  $F$  is large,  $pG_f = pK_2 L_{12} / L_{11}$ . This is the constant value which Curve IV of Fig. 4 reaches and is equal to 3.15.  $pL_{11}$  is therefore 0.23. The constant  $pK_2 L_{12} / L_{11}$  is the  $pK$  of the acid  $d$ . Its value 3.15 is characteristic of a carboxyl group in the same molecule with an imidazole when the amino group is absent. This condition holds in imidazolelactic acid where  $pK_1$  is 2.99 (7).

The agreement is a qualitative confirmation of the absence of the charged amino group in *d*.

Curve VI of Fig. 4 was obtained on the addition of formaldehyde to a histidine solution originally at pH 9.17, the mid-point of the amino group. The equilibria that must be considered are Equations 2, 4, and 5; and the equation for electroneutrality is  $b + e = c + g$ . The extensive changes occur at very low formaldehyde concentrations, in fact below stoichiometric quantity. This introduces a complication in that the total formaldehyde is far removed from the free formaldehyde. However, when  $F$  is large enough, it may be shown that  $pG_{f3} = pK_3 L_{13}/L_{12}$ . This would indicate that Curve VI should reach a minimum constant value. Actually, it has a minimum followed by an increase. The minimum is at  $pG_f = 7.3$ , which is consistent with the formulation of the acid *e*, when compared with imidazolelactic acid which it resembles. The increase of  $pG_f$ , which follows, may be ascribed to solvent influences or to reaction of the imidazole group with formaldehyde as mentioned above. If 7.3 is accepted as  $pK_3 L_{13}/L_{12}$  then  $pL_{13} = -4.5$ . Successive approximations applied to the low formaldehyde concentrations give  $pL_{13} = -4.3$  with considerable uncertainty. The agreement as to order of magnitude is satisfactory.

The behavior of histamine in formaldehyde was studied with the result that Curves III and V of Fig. 4 were obtained. If it is remembered that  $pK_1$  of histamine belongs to the imidazole group, and  $pK_2$  to the amino group (7), and that no carboxyl is present, it is easy to transfer the discussion of histidine to histamine. Here again the minimum pH reached by the amino group (7.2) in Curve V is characteristic of an imidazole uninfluenced by a charged amino group and is comparable to methylimidazole. In the study of Curve III it became evident that in addition to the reactions of Equations 1, 3, and 4, the association of histamine with 2 moles of formaldehyde corresponding to the reaction of Equation 6 had to be considered. The equation for electroneutrality is  $a + h = b + d + e - h$ . When  $h$  is small Equation 16 holds.

$$(16) \quad \left( \frac{G_f}{K_1} - 1 \right) \frac{1}{F} = L_{11} + L_{21} F$$

This equation is plotted in Fig. 5. The intercept is  $L_{11}$  and the slope  $L_{21}$ .  $pL_{11} = -2.59$  and  $pL_{21} = -2.86$ . When  $h$  becomes large, the flattening of the curve to an asymptote at pH 2.4 indicates that  $b$  and  $a$  are present in negligible amounts so that  $h = 0.5(d + e)$ . But  $d + e$  is the total histamine concentration in this case. The observed maximum  $h$  is  $4 \times 10^{-3}$  and that calculated is  $4.17 \times 10^{-3}$ . The association constants for formaldehyde are so great that it can displace  $H^+$  completely from histamine monohydrochloride.

*Stoichiometry*—The stoichiometric behavior of histidine in the formol titration was investigated by Sørensen (10) who found that theoretical values were approached as the end-point became more alkaline. The formaldehyde concentration at the end-point had very little effect. Even under the most favorable conditions histidine was not titrated completely. Henriques and Gjalbak (2) and Kossel and Edlbacher (4) also titrated histidine but obtained values greater than the theory for 1 equivalent. The difference between the results of these authors and those of Sørensen is due to the difference in the initial pH. Sørensen started at the isoelectric point, whereas the others first adjusted the pH to 7. The recent paper of Van Slyke and Kirk (11) discusses the effect of the initial pH on the formol titration. The conditions of the usual formol titration cannot give the desired stoichiometric relation for histidine except through a balancing of errors.

The end-point conditions for histidine are quite different from those of the monoamino acids. The constant of the group that determines the end-point does not change greatly with the formaldehyde concentration when it is large. The most favorable formaldehyde concentration for histidine is at about 0.1 M instead of 2.3 M as advocated for mixtures (6). Calculations based on the principles previously used show the stoichiometric end-point in 2.3 M formaldehyde to be  $10 - 0.5 \log C$ , where  $C$  is the histidine concentration. The error of the titration, when carried to the end-point for mixtures ( $9.6 - 0.5 \log C$ ), is  $2.34/\sqrt{C}$  or in a 0.1 M histidine solution, 7.4 per cent. An error of greater magnitude is involved in the original adjustment to pH 7. Only by an accidental balancing of the errors would the theoretical 1 equivalent be found. If the titration concerns histidine in the absence of

other amino acids, much better results are theoretically possible by adjusting the initial pH to 4, where histidine is all in the form of the monocation, and using only small amounts of formaldehyde, less than 0.5 M. The end-point would then be at 9.5. The advantage in the initial step is that the pH suggested is in the most poorly buffered region of the histidine curve. The amino and imidazole groups are too close together to permit as accurate an adjustment. The use of dilute formaldehyde is to take advantage of the minimum  $pG_i$  shown. It has the further advantage that the amount of alkali used by formaldehyde is diminished so that the end-point is sharper.

#### DISCUSSION

It is a common conception that the titration constants shifted by formaldehyde are to be assigned to amino groups. Aside from the fact that imino groups as in proline also react, the interpretation of formaldehyde shifts must be considered carefully on the basis of such possibilities as are illustrated by histidine and histamine. When a molecule contains other acidic and basic groups not far removed in strength from the amino group, the possibility of internal H ion shifts exists. Only a close study of such cases is likely to lead to correct conclusions about the assignment of constants. Conversely, in the presence of an amino group, a shift in a constant ordinarily assigned to another group does not necessarily imply that the second group reacts with formaldehyde. Internal shifts of H ions may occur and thereby produce changes in the apparent constants.

The constants determining the behaviors of the basic amino acids in the formol titration, according to the unified system presented in the theoretical part, are collected in Table II. Perhaps the most striking thing about these constants is that they are usually much larger than the corresponding constants for the monoamino acids (5).

#### SUMMARY

A unified systematic treatment of the equilibria between dibasic amino acids and formaldehyde has been developed and applied to arginine, histidine, and lysine. Only the amino groups are

considered as reacting with formaldehyde, each of them reacting with 1 or 2 molecules of formaldehyde successively. The stoichiometry of each amino acid in the formol titration is discussed with reference to its equilibria.

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# THE EFFECT OF OXYGENATION AND REDUCTION ON THE EQUILIBRIUM OF HEMOCYANIN WITH ACIDS AND BASES

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Bohr, Hasselbalch, and Krogh discovered in 1904 that carbon dioxide diminished the affinity of hemoglobin for oxygen, a phenomenon commonly known as the "Bohr effect." In 1914, Christiansen, Douglas, and Haldane demonstrated that the reciprocal effect occurs; that blood has a higher carbon dioxide-absorbing capacity in the reduced than in the oxygenated form. It was pointed out by Henderson (1920) that this reciprocal relationship is a physical necessity and could be explained by assuming that combination of hemoglobin with oxygen increased the acidity of one monovalent acid group in the molecule. This explanation was confirmed by Hastings, Van Slyke, Neill, Heidelberger, and Harington (1924).

Redfield, Coolidge, and Hurd reported in 1926 that the normal Bohr effect is reversed in the physiological range in the hemocyanins of *Limulus polyphemus* and *Busycon canaliculatum*. Furthermore, oxygenation decreases the acidity of the *Busycon* blood, while not measurably affecting that of *Limulus*. There is some evidence that the groups involved in the reciprocal relationship are divalent. Redfield, Mason, and Ingalls (1932) have shown that in the production of the colorless form of *Limulus* hemocyanin on the addition of hydrochloric acid, the hemocyanin behaves as a divalent base. The effect of salts and hydrogen ion activity on the oxygen dissociation constant of *Busycon* may be interpreted on the assumption that the group involved is divalent (Redfield and Ingalls, 1932).

This paper describes experiments in which the titration curves of the oxygenated and of the reduced hemocyanins of the lobster,



*Homarus americanus*, and of the horseshoe crab, *Limulus polyphemus*, were determined. From the data, the effect of oxygenation and reduction on the equilibrium of these hemocyanins with acids and bases can be deduced. The number of acidic or basic groups affected by the union of each molecule of oxygen with the hemocyanin is indicated, as well as the magnitude of the change in their dissociation constants. The latter magnitude is related to the degree to which the oxygen dissociation constant may be altered by a change in hydrogen ion activity.

#### EXPERIMENTAL

The hydrogen ion activities of solutions of oxygenated and reduced hemocyanins, to which varying amounts of NaOH and HCl had been added, were determined by the glass electrode. The electrode vessel used resembled that described by Stadie, O'Brien, and Laug (1931). The glass electrodes were constructed according to the method of MacInnes and Dole (1930). The electromotive force from the cell was opposed by one from a Leeds and Northrup type K potentiometer. The point of balance was determined by an electron tube potentiometer similar to that described by Soller (1932).<sup>1</sup> The electrode was standardized over a narrow pH range, just before being used, by plotting the electromotive force given by a buffer against the pH value as determined by the hydrogen electrode. For use in these experiments phosphate and borate buffers were prepared at intervals of 0.4 pH from pH 5.8 to 9.8.

Samples of *Limulus* serum were prepared by adding 10 cc. of water containing varying quantities of NaOH or HCl to 10 cc. of the serum. The mixtures were allowed to stand overnight in the cold before being used. Then each sample was divided into two portions, both of which were completely evacuated in separate tonometers to remove all oxygen and carbon dioxide. Hydrogen was passed into one tonometer and oxygen into the other. They were rotated for 15 minutes in a water bath maintained at 25° to insure that equilibrium was reached. The solutions were forced into the electrode vessel by the excess pressure of gas in the tonometers. At least three consecutive fillings from each

<sup>1</sup> This electron tube potentiometer was designed by and constructed under the supervision of Professor Jeffries Wyman, Jr.

portion were made, and at least three readings taken for each filling. Immediately afterward the same procedure was repeated with the other portion of the sample. The amount of hemocyanin present in the stock solution was calculated from the copper content, as determined by the method of Redfield, Coolidge, and Shotts (1928). Most of the readings on any portion checked within 0.01 pH. The best values, from nine to sixteen in number, were used to compute the average pH. The readings for a typical pair of samples are given below.

*Electromotive Force of Limulus Serum*

Reduced serum				Oxygenated serum			
0.4762	0.4764	0.4761	0.4761	0.4760	0.4763	0.4760	0.4760
0.4761	0.4762	0.4759	0.4760	0.4762	0.4758	0.4760	0.4759
0.4761	0.4761	0.4762	0.4762	0.4759	0.4760	0.4761	0.4761

Purified *Limulus* hemocyanin was prepared by dialyzing serum in the cold under reduced pressure against 0.00001 N NaOH for 3 days, and against 0.00025 N NaOH for 10 days. The dialyzed solution, practically free from salts, was diluted and the hemocyanin was precipitated in the region of its isoelectric point by addition of 0.01 N HCl. The precipitate was separated by centrifuging, redissolved in 0.02 N NaOH, and filtered. Precipitation near the isoelectric point and resolution with NaOH was repeated twice. The resulting solution was kept in the cold and was diluted whenever used. Four determinations of the copper content gave 0.1726, 0.1724, 0.1728, and 0.1704 per cent of the dry weights. These values are in good agreement with that obtained by Redfield, Coolidge, and Shotts (0.173 per cent), and indicate that the solution used was pure. The samples were prepared by adding 10 cc. of water, containing various quantities of HCl or NaOH, to 5 cc. of the hemocyanin solution. The resulting concentration was 2.114 gm. of protein per 100 cc. The preparation employed contained  $9 \times 10^{-6}$  moles of base per gm. of hemocyanin as determined by the quantity of dilute HCl required to produce maximal precipitation in the region of the isoelectric point. This quantity was added to the NaOH (or subtracted from HCl) mixed with the sample in estimating the amount of base in the samples. The experimental procedure described for the serum was then followed.

The range covered was pH 6.2 to 10. In more acid solutions the protein is precipitated.

Lobster serum was diluted with an equal volume of water containing various quantities of acid or base. The concentration of hemocyanin, calculated from the copper content as determined in this laboratory (0.173 per cent), was 3.51 gm. in 100 cc. of the serum.<sup>2</sup>

Possible sources of error and the methods used to eliminate them are listed below.

*Temperature Fluctuation*—The temperature was regulated to 0.1°. Since a temperature change of 25° causes a maximum pK shift of 0.86 pH unit (Cohn, 1931), the error due to temperature fluctuation would be about 0.0034 pH unit.

*Changes in Cell Constant*—This is likely to be very small. However, the number of readings taken and the rechecks eliminate it as a serious factor.

*Gaseous Interchange in Electrode Vessel*—Stadie, O'Brien, and Laug (1931), using the same type of electrode vessel, studied the change in pH of serum equilibrated with 40 mm. of carbon dioxide due to loss of the gas during the measurement. In one case the air in the pipette was first swept out by carbon dioxide; in the second case the operation was omitted. There were no differences in the values obtained. I was not able to detect any definite drifts during the measurements made upon lobster serum, a blood in which oxygenation and reduction affects the pH.

*Effect of Na above pH 9.5*—MacInnes and Dole (1930) report that the pH values calculated by their equation deviate about 0.02 unit from the hydrogen electrode values at pH 9.5 in the presence of 0.1 N Na. These deviations, which increase rapidly above pH 9.5, are due to the fact that the system is also functioning as a sodium electrode. Although the electrode was standardized with buffers above pH 9.5, a small error might be introduced in the absolute value of the pH due to differences in sodium concentration of hemocyanin solutions and buffers. Since no correction has been made for this factor, it is probable that the pH values shown above 9.5 are slightly in error. No significant conclusions have

<sup>2</sup> Hernler and Philippi (1933) report a copper content of 0.187 per cent. If their value is used, the concentration of hemocyanin is 3.25 gm. per 100 cc. of serum.

been drawn involving the absolute value of the pH measurements above this limit.

### Results

The results obtained with both the serum and dialyzed hemocyanin solution of *Limulus polyphemus* are recorded in Tables I and II. They show that the pH is not affected by oxygenation

TABLE I

*pH Values of Oxygenated and Reduced Samples of Limulus Serum*

Concentration = 2.15 gm. per 100 cc.; 1 cc. of 0.1 N HCl or NaOH =  $23.3 \times 10^{-6}$  moles per gm. of hemocyanin; temperature = 25°.

Base added*	Base added	pH reduced	pH oxygenated
cc.	$10^{-5}$ moles per gm.		
-1.0	-23.3	6.530	6.522
-0.75	-17.46	6.780	6.731
-0.5	-11.65	7.000	6.988
-0.4	-9.32	7.111	7.120
-0.2	-4.66	7.282	7.291
-0.1	-2.33	7.420	7.421
0.0	0.0	7.480	7.480
0.2	4.66	7.690	7.700
0.4	9.32	7.850	7.830
0.5	11.65	8.060	8.075
0.6	13.98	8.261	8.262
0.7	16.31	8.410	8.391
1.0	23.30	8.710	8.720
1.4	32.62	9.232	9.230
2.0	46.60	9.600	9.600
2.5	58.20	9.961	9.960

\* Minus values indicate addition of HCl.

and reduction in the pH range 6.51 to 9.96. The difference between any two points in a pair is in practically every case 0.02 pH or less, and in most cases 0.01 pH or less. Since these differences show no definite direction and are within the experimental error, it may be stated that the acid and basic properties of *Limulus* hemocyanin are not significantly changed by combination with oxygen. This is in agreement with Redfield, Coolidge, and Hurd's report (1926) that oxygenation and reduction had no effect on the carbon dioxide-combining capacity of *Limulus* hemocyanin.

TABLE II

*pH Values of Oxygenated and Reduced Samples of Purified Hemocyanin*

Concentration = 2.114 gm. per 100 cc.; base bound =  $9 \times 10^{-5}$  moles per gm.; 1 cc. of 0.1 N HCl or NaOH =  $31.53 \times 10^{-5}$  moles per gm.; temperature = 25°.

Base added*	Base present	pH reduced	pH oxygenated
cc.	$10^{-5}$ moles per gm.		
-0.2	2.7	6.241	6.250
-0.1	5.85	6.413	6.404
0	9.0	6.701	6.700
0.1	12.15	6.850	6.821
0.2	15.3	7.112	7.110
0.3	18.45	7.281	7.290
0.4	21.6	7.461	7.432
0.5	24.75	7.691	7.700
0.6	27.9	7.891	7.891
0.7	31.05	8.120	8.101
0.8	34.2	8.310	8.320
1.0	40.5	8.811	8.112
1.2	46.8	9.152	9.170
1.4	53.1	9.781	9.772

\* Minus values indicate addition of HCl.

TABLE III

*pH Values of Oxygenated and Reduced Samples of Serum of Homarus americanus*

Concentration = 0.755 gm. per 100 cc.; 1 cc. of 0.1 N HCl or NaOH =  $56.9 \times 10^{-5}$  moles per gm.; temperature = 25°.

Acid added	Acid added	pH reduced	pH oxygenated
cc.	$10^{-5}$ moles per gm.		
1.5	85.3	6.151	6.151
1.25	71.1	6.731	6.690
1.00	56.9	7.155	7.095
0.8	45.5	7.411	7.372
0.65	37.0	7.570	7.531
0.4	22.86	7.845	7.820
0.2	11.48	8.090	8.071
0	0	8.520	8.510

It is shown in Table III that the oxygenated serum of *Homarus americanus*, the lobster, is more acid than the reduced. The differences in the base-binding capacity of the oxygenated and reduced forms at various pH values can be determined by plotting graphs of these data. The difference in base-binding power occurs over the range pH 6.2 to 8.4 with a maximum of  $0.23 \times 10^{-5}$  moles per gm. at pH 7.25. The copper content of lobster hemocyanin is  $0.29 \times 10^{-5}$  moles per gm. Thus, combination of the hemocyanin with 1 atom of oxygen has increased the base-binding power by 0.758 equivalent.

The data of the experiments described above and of other similar experiments have been analyzed to determine how many and what types of acid or basic groups are involved, and to estimate the magnitudes of the shifts in the dissociation constants. The graphical method described by Hastings *et al.* (1924) was used in this treatment.

Fig. 1 shows the equivalents of acid or base displaced from a monovalent group by shifts of the magnitude  $pK_0 - pK_1$ . The curves were constructed from the formula

$$\Delta B = \frac{1}{1 + 10^{pK_0 - pH}} - \frac{1}{1 + 10^{pK_1 - pH}}$$

Fig. 2 was constructed on the assumption that the addition of 1 atom of oxygen caused a simultaneous shift in two monovalent groups. The equation is

$$\Delta B = 2 \left( \frac{1}{1 + 10^{pK_0 - pH}} - \frac{1}{1 + 10^{pK_1 - pH}} \right)$$

Fig. 3 was constructed for a divalent group from the formula

$$\Delta B = \frac{1}{1 + 10^{pK_0 - 2pH}} - \frac{1}{1 + 10^{pK_1 - 2pH}}$$

Analysis of the three equations shows that in each case the maximum value of  $\Delta B$  occurs when  $pH = (pK_1 + pK_0)/2$ . For each of the graphs  $pK_0$  was arbitrarily set equal to 0. The curves can be made to correspond to the pH range of the experiments by adding the appropriate quantity to the pH values of Figs. 1 to 3. By a comparison of the experimental results with the curves of

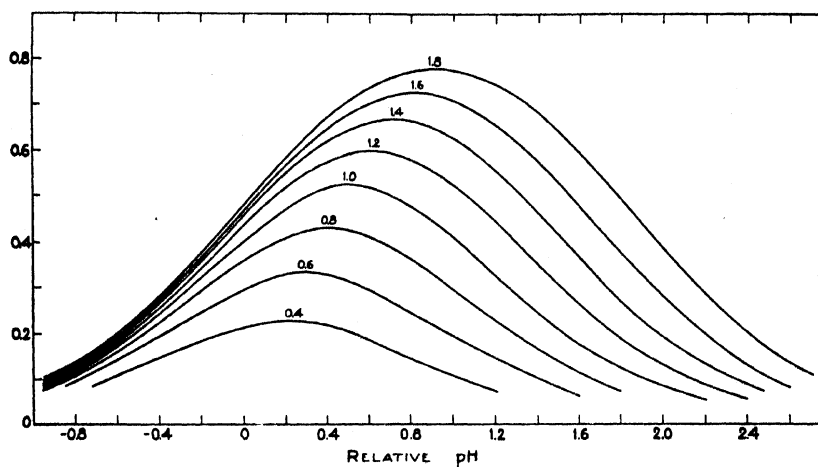


FIG. 1. The equivalents of acid or base,  $\Delta B$ , displaced from a single monovalent group by increase of the pK value of the magnitude indicated over each curve; at pH values measured relative to the pK characteristic of the initial state of the group.

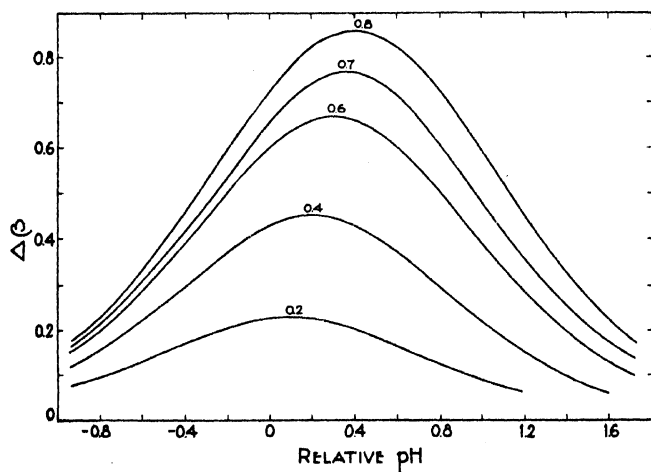


FIG. 2. The equivalents of acid or base,  $\Delta B$ , displaced simultaneously from two monovalent groups; at pH values measured relative to the pK characteristic of the initial state of the group.

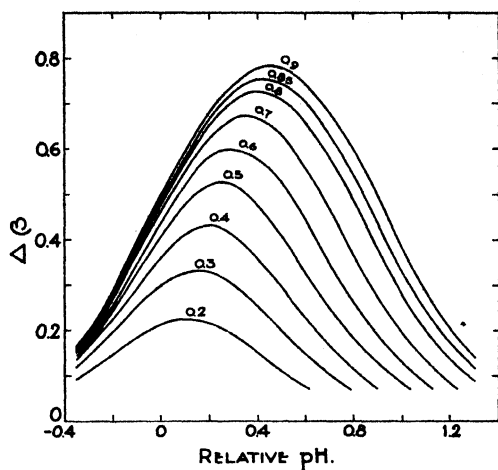


FIG. 3. The equivalents of acid or base,  $\Delta B$ , displaced from a single divalent group; at pH values measured relative to the pK characteristic of the initial state of the group.

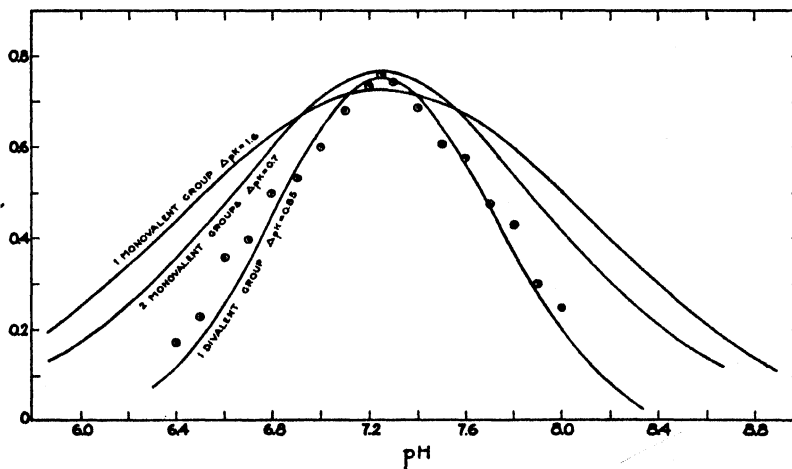


FIG. 4. Comparison of the change in the base bound by lobster hemocyanin as the result of reduction with that estimated for monovalent and divalent groups.



Figs. 1 to 3 (see Fig. 4), it is possible to identify the number and kind of groups, and to estimate  $\Delta pK$ .

*Comparison of Effect of Oxygenation on Titration Curves of Various Respiratory Proteins*

*Homarus americanus*—An inspection of Fig. 4 indicates that a divalent group is involved and that its dissociation constant is decreased by about 0.85 pH unit by the oxygenation of the hemocyanin. Obviously the pH range over which the difference occurs in the case of the experimental data is too small to correspond to that characteristic of monovalent groups.

*Limulus polyphemus*—Since the reaction of the *Limulus* blood is not measurably affected by oxygenation, there is no detectable shift in the dissociation constant.

*Maia squinado*—Kerridge (1926) used carbon dioxide as acid and determined the value of pH by means of a glass electrode. The maximum value of  $dB/dHcyCuO$  is about 0.55. If it is assumed that the group involved is divalent, the shift in dissociation constant is about 0.5 to 0.6 pH unit. However, the data are not sufficiently precise to make such a conclusion certain.

*Helix pomatia*—Roche (1932) has studied the effect of oxygenation on the titration curves of the dialyzed hemocyanin of *Helix pomatia*. The oxygenated form is a stronger acid from pH 6.5 to 8.0 and a weaker acid from pH 8.0 to 9.5. The maximum values of  $dB/dHcyCuO$  are  $\pm 0.7$ . These figures correspond more closely to divalent groups shifting by about 0.8 pH unit than to the other types. Roche has estimated that oxygenation shifts the dissociation constant of the titration curve by 0.6 to 0.7 pH unit.

*Hemoglobin*—Carbon dioxide absorption curves were determined on oxygenated and reduced solutions of purified horse hemoglobin at 38° by Hastings *et al.* (1924). The pH was calculated from the Henderson-Hasselbalch equation. From results obtained over the pH range 6.8 to 7.6, the oxygenated hemoglobin was found to be a stronger acid. The maximum value for  $dB/dHbFeO_2$  was 0.66 and occurred at pH 7.6. pH was estimated to be 1.46 pH units for a monovalent group.

#### SUMMARY

1. The titration curves of the oxygenated and reduced forms of *Limulus* serum, dialyzed *Limulus* hemocyanin, and *Homarus* serum have been determined.

2. Oxygenation does not measurably change the hydrogen ion activity of either the serum or dialyzed hemocyanin of *Limulus*.

3. The addition of oxygen makes the serum of *Homarus americanus* more acidic. The maximum difference in base bound by the oxygenated and reduced forms is 0.758 equivalent for each atom of oxygen.

4. Analysis has indicated that oxygenation decreases the dissociation constant of a divalent acid or basic group by about 0.8 to 0.9 pH unit.

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# THE INFLUENCE OF EPINEPHRINE ON THE PURINE METABOLISM OF ORDINARY AND DALMATIAN BREEDS OF DOGS\*

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In two previous communications it was shown that the subcutaneous injection of insulin led to an increased excretion of allantoin in the dog of ordinary breed (1), whereas in the Dalmatian this hormone effected a rise in uric acid of the blood as well as in the excretion of this substance in the urine (2). In the latter breed it was found that a time interval of at least 1 hour elapsed before the blood uric acid began to rise, although the blood sugar had fallen to low levels by that time. It seemed a reasonable inference, therefore, that the rise in uric acid was secondary to the hypoglycemia rather than a direct effect of insulin. This was later shown to be the case when it was observed that the rise in blood uric acid did not occur if glucose sufficient to prevent the fall in blood sugar was administered just prior to the insulin. In view of the well known action of hypoglycemia in releasing epinephrine from the adrenal gland, it became of interest to consider the possibility that it was by way of epinephrine that insulin affected the purine metabolism, or, in other words, that the epinephrine was the direct stimulus to the rise in blood uric acid observed under the influence of insulin. Although an attractive suggestion, this hypothesis lacked satisfactory evidence to substantiate or refute it. To supply the deficiency in some measure has been the purpose of the present investigation. It has been claimed that epinephrine is capable of affecting purine metabolism in various species of animals (3-12), but the results reported

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have not been uniform. Moreover, no studies dealing with the effects of this hormone on the Dalmatian coach-dog have as yet appeared. In order to determine the interplay of the two hormones, insulin and epinephrine, in relation to purine metabolism, a study was, therefore, made of the influence of epinephrine upon the purine constituents of blood and urine in the same breeds of dogs, namely the dog of ordinary breed and the Dalmatian, and under conditions comparable to those previously reported by us for insulin (1,2).

### EXPERIMENTAL

Dogs of ordinary breed and Dalmatian coach-dogs were used in this study. In the former, the effect of epinephrine on the excretion of allantoin was investigated; the care of the animals and the methods of analysis were identical with those previously used in the study of the effects of insulin on this breed. In the case of the Dalmatians, the influence of epinephrine on the excretion of allantoin and uric acid in the urine and on the occurrence of uric acid in the blood was investigated, and here again the methods employed in the study of insulin in Dalmatians (2) were adopted in all details. In the urinary studies, 4 days of a nitrogen equilibrium in which the daily excretion of total nitrogen did not vary by more than 0.20 gm. were accepted as a control period prior to the injection of epinephrine.

All injections of epinephrine were made subcutaneously. With one exception, the dose employed was in the neighborhood of 0.10 mg. per kilo of body weight, a dose which, when given to the dog in this manner, has been shown to be insufficient to raise the blood pressure (13).

### Results

*Effect of Epinephrine on Excretion of Allantoin by the Dog of Ordinary Breed*—Four animals were injected with the hormone and an abbreviated form of the results obtained on two of these, namely Dogs E and T, is shown in Table I. With the exception of Dog T, which received two injections of the hormone—the first to the amount of 0.20 mg. per kilo of body weight—these animals received subcutaneously 0.10 mg. of epinephrine per kilo of body weight. During the 5 hour period that followed the injection, the

allantoin excretion of all four dogs rose markedly as compared with the elimination during the corresponding 5 hours of the

TABLE I

*Effect of Epinephrine on Excretion of Allantoin by Dogs of Ordinary Breed*

	Day of experiment	Allantoin N			Urine volume	
		19 hrs.	5 hrs.	24 hrs.	19 hrs.	5 hrs.
		gm.	gm.	gm.	cc.	cc.
Dog E	3	0.165	0.032	0.197	130	45
	4	0.150	0.035	0.185	130	50
	5	0.162	0.087	0.249	130	45
	6	0.180	0.037	0.217	145	25
	7	0.145	0.035	0.180	150	35
Dog T	2	0.109	0.028	0.137	115	25
	6	0.121	0.026	0.147	120	25
	7	0.120	0.089	0.209	180	15
	8	0.136	0.025	0.161	130	50
	9	0.118	0.031	0.149	120	30
	13	0.115	0.029	0.144	125	30
	14	0.118	0.029	0.147	125	30
	15	0.110	0.060	0.170	130	25
	16	0.105	0.029	0.134	130	25
	17	0.114	0.030	0.144	125	25

*Dog E*—This dog was catheterized at 7.30 a.m. and 12.30 p.m. daily; fed at 12.40 p.m. daily. Diet: 130 gm. of diet mixture + 22 gm. of lard + 11 gm. of unsalted butter. Diet N = 5.85 gm. per 100 gm. of diet mixture. On the 5th day 0.10 mg. of epinephrine per kilo of body weight was injected subcutaneously at 7.30 a.m. The period of 19 hours represents the interval from 12.30 p.m. to 7.30 a.m.; the period of 5 hours, 7.30 a.m. to 12.30 p.m. The dog weighed 12.8 kilos.

*Dog T*—This dog was catheterized at 7.30 a.m. and 12.30 p.m. daily; fed at 12.40 p.m. daily. Diet: 110 gm. of diet mixture + 20 gm. of lard + 10 gm. of unsalted butter. Diet N = 5.85 gm. per 100 gm. of diet mixture. On the 7th day 0.20 mg. of epinephrine per kilo of body weight was injected subcutaneously at 7.30 a.m.; on the 15th day 0.10 mg. of epinephrine per kilo of body weight was injected subcutaneously at 7.30 a.m. The period of 19 hours represents the interval from 12.30 p.m. to 7.30 a.m.; the period of 5 hours, from 7.30 a.m. to 12.30 p.m. The dog weighed 11.5 kilos.

previous day. Thus the allantoin nitrogen excreted during the 5 hour period on the day preceding the injection was 26, 35, 29, and 29 mg. for Dogs A, E, S, and T respectively, whereas during

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the corresponding 5 hours following the administration of epinephrine (0.10 mg. per kilo of body weight) these animals excreted 59, 87, 53, and 60 mg. respectively—increases ranging from 83 to 150 per cent. Dog T, on the occasion when it received the larger dose of epinephrine, excreted 89 mg. of allantoin nitrogen, an increase of 240 per cent above the amount excreted in the previous control period.

In all dogs except Dog E, the elimination of the extra allantoin produced by the hormone was complete by the end of 5 hours when the amount of epinephrine injected was 0.10 mg. per kilo of body weight. On the single occasion when Dog T received twice this quantity of the hormone, an increased elimination of allantoin was also found in the next (19 hour) period.

Despite the extra output of allantoin during the first 5 hours following the injection of epinephrine, no change in urine volume was found for this period.

*Effect of Epinephrine on Excretion of Allantoin and Uric Acid by the Dalmatian Coach-Dog*—Three Dalmatians received epinephrine injections and an abbreviated form of the results obtained on two of these animals, namely Dogs P and S-S, is shown in Table II. A pronounced increase in the output of uric acid by all animals was observed after the subcutaneous injection of approximately 0.10 mg. of epinephrine per kilo of body weight. The greater elimination of uric acid occurred during not only the first 5 hours following the administration of the hormone, but the next 19 as well. In two of the three dogs, namely Dogs D-S and P, the major portion of the extra uric acid was found in the first 5 hour sample of urine.

An increased elimination of allantoin also followed the injection of epinephrine. Dog P responded by approximately doubling its normal elimination during the first 5 hours and by significantly increasing this amount during the subsequent 19 hours. The most marked effect was found in Dog S-S, in which the output of allantoin rose 162 per cent during the first 5 hours. In this animal, moreover, the output of allantoin was significantly increased in the next 19 hours. The least effect was observed in Dog D-S, which nevertheless showed a 33 per cent increase in its elimination of allantoin in the 5 hour sample of urine.

An increase in urinary volume, varying in degree in different

**TABLE II**  
*Effect of Epinephrine on Excretion of Allantoin and Uric Acid by Dalmatian Dogs*

	Day of experiment	Allantoin N			Uric acid N			Urine volume	
		19 hrs.	5 hrs.	24 hrs.	19 hrs.	5 hrs.	24 hrs.	19 hrs.	5 hrs.
		gm.	gm.	gm.	gm.	gm.	gm.	cc.	cc.
Dog P	1	0.076	0.019	0.095	0.127	0.038	0.165	500	200
	2	0.077	0.020	0.097	0.139	0.035	0.174	800	350
	6	0.078	0.016	0.094	0.140	0.029	0.169	600	100
	7	0.078	0.038	0.116	0.140	0.097	0.237	600	300
	8	0.090	0.022	0.112	0.160	0.042	0.202	700	150
	9	0.079	0.020	0.099	0.106	0.035	0.141	550	250
	10	0.080	0.020	0.100	0.139	0.032	0.171	550	150
	1	0.093	0.018	0.111	0.123	0.045	0.168		70
	2	0.096	0.017	0.113	0.101	0.042	0.143		65
Dog D-S	3	0.090	0.017	0.107	0.111	0.047	0.158		70
	4	0.092	0.035	0.127	0.102	0.086	0.188		250
	5	0.110	0.021	0.131	0.137	0.032	0.169		110
	6	0.089	0.016	0.105	0.112	0.046	0.158		75
	1	0.090	0.017	0.107	0.111	0.017	0.128		70
	2	0.091	0.015	0.106	0.110	0.016	0.126		80
Dog S-S	3	0.094	0.016	0.110	0.117	0.021	0.138		80
	4	0.089	0.042	0.131	0.106	0.046	0.152		140
	5	0.108	0.020	0.128	0.126	0.032	0.158		80
	6	0.087	0.016	0.103	0.105	0.022	0.127		85

*Dog P*—This dog was catheterized at 9.00 a.m. and 2.00 p.m. daily; fed at 2.10 p.m. daily. Diet: 210 gm. of diet mixture + 38 gm. of lard + 14 gm. of unsalted butter. Diet N = 5.85 gm. per 100 gm. of diet mixture. On the 7th day 0.10 mg. of epinephrine per kilo of body weight was injected subcutaneously at 9.00 a.m. The period of 19 hours represents the interval from 2.00 p.m. to 9.00 a.m.; the period of 5 hours, from 9.00 a.m. to 2.00 p.m. The dog weighed 18.0 kilos.

*Dog D-S*—This dog was catheterized at 8.00 a.m. and 1.00 p.m. daily; fed at 1.10 p.m. daily. Diet: 210 gm. of diet mixture + 38 gm. of lard + 19 gm. of unsalted butter. Diet N = 5.78 gm. per 100 gm. of diet mixture. On the 4th day 2.0 mg. of epinephrine were injected subcutaneously at 8.10 a.m. and 0.09 gm. of glucose was excreted during the 5 hour interval; on the 5th day 0.68 gm. of glucose was found in the 19 hour sample of urine. The period of 19 hours represents the interval from 1.00 p.m. to 8.00 a.m.; the period of 5 hours, from 8.00 a.m. to 1.00 p.m. The dog weighed 19.1 kilos.

*Dog S-S*—This dog was catheterized daily at 8.05 a.m. and 1.05 p.m.; fed daily at 1.10 p.m. Diet: 200 gm. of diet mixture + 36 gm. of lard + 18 gm. of unsalted butter. Diet N = 5.78 gm. per 100 gm. of diet mixture. On the 4th day 2.0 mg. of epinephrine were injected subcutaneously at 8.10 a.m. and 0.06 gm. of glucose was excreted during the 5 hour interval; on the 5th day 0.29 gm. of glucose was found in the 19 hour sample of urine. The period of 19 hours represents the interval from 1.05 p.m. to 8.05 a.m.; the period of 5 hours, from 8.05 a.m. to 1.05 p.m. The dog weighed 19.3 kilos.



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experiments, was observed in the 5 hour period that followed the administration of epinephrine.

*Effect of Epinephrine on Uric Acid of Blood of the Dalmatian Coach-Dog*—The changes in blood uric acid that followed the subcutaneous injection of 0.10 mg. of epinephrine per kilo of body weight in three of the four dogs studied are shown in Table III.

TABLE III  
*Effect of Epinephrine on Blood Uric Acid of Dalmatian Coach-Dogs*

	Weight	Time	Blood uric acid	Blood sugar
	kg.	min.	mg. per cent	mg. per cent
Dog P	18.5	0	0.44	71
		3	1.8 mg. epinephrine subcutaneously	
		60	0.82	105
		120	1.78	136
		178	2.40	148
		244	2.75	152
		341	1.95	118
		459	0.64	88
Dog D-S	20.5	0	0.38	81
		2	2.0 mg. epinephrine subcutaneously	
		54	0.63	91
		104	1.05	136
		167	1.32	150
		224	1.14	127
		312	0.88	109
		433	0.68	98
Dog S-S	20.0	0	0.43	79
		3	2.0 mg. epinephrine subcutaneously	
		53	0.92	99
		100	1.83	120
		164	2.00	127
		221	1.73	120
		308	1.23	107
		429	0.89	95

A rise occurred in all animals. The normal postabsorptive values fluctuated between 0.38 and 0.50 mg. per 100 cc. of blood, whereas the maximum values obtained after the injection of the hormone were 2.75, 2.00, 2.00, and 1.32 mg. per cent in Dogs P, S-S, L-A, and D-S respectively, the latter values representing increases between 250 and 530 per cent above the normal. At the end of

the 1st hour after the administration of epinephrine, rises in uric acid ranging from 66 to 110 per cent of the normal values were observed in the blood of these dogs. The highest values were found between 2.7 and 4 hours after the administration of the hormone. The blood uric acid then slowly descended and remained well above the normal level even after 7 hours in three of the four animals studied. In Dog L-A normal values were regained after 7.5 hours.

As control experiments, three of the Dalmatian dogs were injected subcutaneously with 2 cc. of sterile 0.8 per cent saline. No significant changes were found in the uric acid content of the blood during the 7 or 8 hours following the injection of the saline.

#### DISCUSSION

Epinephrine, administered subcutaneously in amounts that produce no change in blood pressure, has a striking effect on the purine metabolism of the dog of ordinary breed and on the Dalmatian-coach-dog. In the former, the influence of this hormone was demonstrated upon allantoin, a pronounced increase in the excretion of this substance in the urine having been observed on five different occasions in four animals. In the Dalmatian coach-dog, epinephrine led to a marked rise in the elimination of uric acid and allantoin in four experiments on three animals. In the latter breed a varying degree of diuresis accompanied the increased excretion, but this can be definitely ruled out as the factor responsible for the presence of the augmented amounts of uric acid in the urine, since there was a considerable rise also in the blood uric acid, a rise prolonged for well over 7 hours after the administration of the epinephrine. In previous studies (1, 2) no relation was found between urinary volume and the amount of allantoin excreted. Thus, in the case of the dog of ordinary breed, insulin effected a rise in the excretion of allantoin, while the urine volume remained constant; in the Dalmatian, on the other hand, this hormone led to an increase in urine volume but to no alteration in the amount of allantoin eliminated. It seems a reasonable inference, therefore, that the augmented output of allantoin observed under the influence of epinephrine was not the result of the accompanying diuresis.

It has been previously demonstrated that insulin is capable of

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increasing the concentration of uric acid in the blood of the Dalmatian coach-dog (2). Although 3 hours after the injection of insulin the blood uric acid reached a value 3 times that of the normal level, this constituent of the blood was not significantly altered at the 1 hour interval following the administration of the hormone. This delay in the change of the blood uric acid is apparently a characteristic phenomenon in the action of insulin, for it was observed with remarkable regularity in the four Dalmatians studied. It is worthy of note, therefore, that no such delayed response as regards epinephrine was found in this breed of dog. At the end of the 1st hour the blood uric acid had already risen significantly, in one case to a level 110 per cent above normal. The response of blood uric acid to epinephrine is probably an immediate one.

A close correlation between the duration of the hyperuric-acidemia and the presence of increased amounts of uric acid in the urine was found in the Dalmatian coach-dog after the administration of epinephrine. Although observations on blood and urine were not made simultaneously, approximately the same doses of epinephrine were employed throughout in this breed of dog. The major portion of the extra uric acid that made its appearance in the urine as a result of the injection of epinephrine was excreted during the first 5 hours, at the end of which time the peak in the rise of the blood uric acid had already been passed. In all animals, however, the uric acid of the blood was still above normal for several hours after the end of the 5 hour period, and this fact accounts for the smaller but nevertheless significant amounts of extra uric acid that appeared in the subsequent 19 hour sample of urine.

The purpose of the present investigation was to determine whether the effects of insulin on purine metabolism (1, 2) could be explained by a reflex stimulation of epinephrine. Whether or not such a relation has been demonstrated can best be gaged by a comparison of the various effects of the two hormones on the purine metabolism of the breeds studied:

1. In the dog of ordinary breed, both hormones brought about an increased excretion of allantoin in the urine.
2. In the Dalmatian coach-dog, both hormones led to a rise in the uric acid content of the blood, but whereas in the case of

epinephrine the hyperuricacidemia was already present 1 hour after its injection, no change in blood uric acid was produced with insulin in the same amount of time. At the end of this interval, however, the blood sugar had already fallen, and the delayed response of uric acid after the administration of insulin might, therefore, be accounted for by the assumption that this amount of time was required for the hypoglycemia to elicit epinephrine, which is the real stimulus for the hyperuricacidemia that follows the administration of insulin. The fact, moreover, that no such delay followed the injection of epinephrine is in harmony with this view.

3. Both hormones effect a marked rise in the excretion of uric acid in the Dalmatian coach-dog. Although insulin was without influence upon urinary allantoin in this breed, epinephrine on the other hand increased its excretion in the urine. Despite the fact that the significance of this difference in the behavior of allantoin as regards the two hormones must remain unexplained at present, the rest of the evidence is consistent with the view that insulin hypoglycemia influences purine metabolism by way of the epinephrine secreted.

#### SUMMARY

1. To determine whether the effects of insulin on purine metabolism previously reported for the dog of ordinary breed and for the Dalmatian coach-dog could be explained by a secretion of epinephrine induced by hypoglycemia, a study was made of the influence of epinephrine upon purine derivatives in either blood or urine or both in these two breeds.

2. In normal dogs, epinephrine, administered in quantities insufficient to alter the blood pressure, led to an increased excretion of allantoin in the urine.

3. In pure bred Dalmatians, epinephrine produced a considerable rise in the uric acid of the blood, an effect that was present to a marked degree at the end of the 1st hour and was sustained for well over 7 hours after the injection of 0.10 mg. of the hormone per kilo of body weight. An augmented elimination of uric acid and of allantoin in the urine of these dogs also occurred under the influence of epinephrine.

4. The interrelation of insulin and epinephrine in respect to purine metabolism is briefly discussed.

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# THE DETERMINATION OF OXYGEN IN BLOOD IN THE PRESENCE OF ETHER BY A MODIFICATION OF THE VAN SLYKE-NEILL TECHNIQUE

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The necessity for determining the oxygen content and capacity of blood under ether anesthesia is one which frequently arises, yet no reliable method for making such determinations has been published. Peters and Van Slyke (1) in their recent book made note of this omission and suggested that oxygen might presumably be determined by the same principle evolved by Austin (2) for determining  $\text{CO}_2$  in blood serum which contained ether. Austin found that the presence of ether in serum caused an error of about 15 per cent in excess of true  $\text{CO}_2$  values. This was due to the fact that much of the ether extracted from the acid solution and measured with the mixed gases was reabsorbed by the NaOH during the absorption of  $\text{CO}_2$  and thus became absent from the final measurement. He eliminated the error by reextraction of the alkaline solution after the absorption of  $\text{CO}_2$  with alkali. An experimentally derived correction factor was then used to interpolate for the change in solubility. Fuss and Derra (3) slightly modified Austin's technique in an attempt to determine both  $\text{CO}_2$  and  $\text{O}_2$  in the same sample of blood in the presence of ether with results apparently satisfactory to themselves.

The present communication resulted from an attempt to measure the oxygen content of blood of individuals under ether anesthesia. Preliminary work with both the Austin (2) and Fuss and Derra (3) modifications of the Van Slyke-Neill technique proved unsatisfactory as will be shown below.

The method finally developed depends upon the removal of the blood-reagent mixture, in which most of the ether is in solution, from the chamber of the machine so that the liberated oxygen may be measured directly without interference from the ether.

### Method

*Reagents*—The usual reagents of the Van Slyke-Neill procedure for oxygen are used without any changes (1, 4).

*Apparatus*—The Van Slyke-Neill manometric extraction apparatus was used (4), with a modified Hempel pipette (5) filled with an air-free solution of equal parts of a saturated sodium chloride solution and glycerol or with mercury.<sup>1</sup> The glycerol-salt solution is protected from the air by a layer of paraffin oil.

The analysis consists of the following steps.

1. The gases CO<sub>2</sub>, O<sub>2</sub>, and N<sub>2</sub> and part of the ether are extracted from the blood sample in the chamber of the Van Slyke-Neill apparatus.

2. The mixture of gases is transferred to the Hempel pipette for storage during the following step.

3. The blood and reagent mixture is removed from the extraction chamber and replaced by air-free water.

4. The mixture of gases is returned from the Hempel pipette to the extraction chamber.

5. CO<sub>2</sub> is absorbed by the addition of 1 N NaOH and the gases reextracted until checked pressure readings are obtained.

6. O<sub>2</sub> is absorbed by the addition of sodium hydrosulfite and the pressure reading of the gas taken.

The details of the successive steps are given below, but the reader is referred to the original authors for more complete descriptions.

The directions are given for 1 cc. samples of blood.

### Procedure

*Extraction of Gases from Blood Sample*—The Van Slyke-Neill procedure (1, 4) for oxygen is used without modification up to the

<sup>1</sup> The technique was originally worked out by the authors using mercury in the Hempel pipette. It was suggested by Dr. Van Slyke that the substitution of a solution of equal parts of saturated sodium chloride and glycerol for mercury would work as efficiently and possibly in addition absorb the remaining quantity of interfering ether. The results as described in the experimental part of this communication indicate that of the two media, mercury is slightly more accurate but, due to its weight, is more difficult to handle than glycerol-salt solution. The recovery of oxygen with either means is within an average error of less than 1 per cent.

completion of the extraction of oxygen. At this point 1 N NaOH is not added to absorb  $\text{CO}_2$  but instead the gases are transferred to the Hempel pipette.

*Transfer of Gases to Hempel Pipette*—When the extraction is completed, the gases are transferred to the Hempel pipette and stored over air-free glycerol-salt solution or mercury while the chamber is cleaned and air-free water substituted for the blood-reagent mixture. The mechanical manipulation of apparatus and stop-cocks during the process of transfer is essentially the same as that of Sendroy and Liu (6).

The following modification of the above method may be suggested for the transferring of gases to the Hempel pipette. When the blood-reagent mixture from the extraction apparatus has passed slightly beyond the bore of stop-cock *b* (cf. (6) Fig. 1), the stop-cock *b* is closed and turned one-half revolution so that the chamber connects with the side arm. The chamber may then be emptied of the solution through the side arm which is connected by a rubber tube to a receptacle. When the chamber is freed of the reagent mixture, the stop-cock connecting the leveling bulb is closed. Stop-cock *b* is turned back to the original position connecting the Hempel pipette and the Van Slyke-Neill apparatus. The stop-cock connecting the leveling bulb is cautiously opened and the gases followed by the small amount of solution and mercury are passed into the Hempel pipette slightly beyond stop-cock *a*. Stop-cocks *b* and *a* are closed and the Hempel pipette is set aside while the chamber is cleaned.

*Replacement of Blood and Reagent Mixture by Air-Free Water*—If the above modification is not used, the blood and reagent solution are removed from the chamber. The apparatus is cleaned by admitting 1.0 or 2.0 cc. of approximately 1 N NaOH and 10 cc. of distilled water and shaken for 1 minute. The alkaline solution is ejected and the chamber rinsed with distilled water. 1 drop of caprylic alcohol and 5.0 cc. of water are admitted and rendered air-free by extracting in the evacuated chamber for 3 minutes. The extracted air is expelled and 1.5 cc. of water are run into the cup, leaving 3.5 cc. in the chamber. Stop-cock *b* is now closed and the apparatus is ready to receive the gases from the Hempel pipette.



*Transfer of Gases from Hempel Pipette to Extraction Chamber*—The gases are transferred from the Hempel pipette to the extraction chamber after the manner of Sendroy and Liu (6).

*Absorption of CO<sub>2</sub> with NaOH*—The volume of the gases is adjusted to about 5 cc. by withdrawing mercury from the chamber. 2 cc. of air-free 1 N NaOH are placed in the cup, of which 1 cc. is slowly admitted to the chamber (1, 4). The stop-cock is sealed with mercury and the chamber is evacuated to the 50 cc. mark and reextracted for 3 minutes. Manometer reading  $p_1$  is taken at the 2 cc. mark. The reextraction is repeated for 1 minute until the reading checks within a few tenths of a mm.

Following the admission of the NaOH, checks are usually obtained with the first two reextractions and in the majority of cases there is rarely a difference greater than 1 mm., so that reliance upon one reextraction will introduce no great error. However, for absolute accuracy reextraction until checks are obtained is recommended.

*Absorption of O<sub>2</sub> with Hydrosulfite*—The gas volume is adjusted to about 5 cc.; 2 cc. of air-free alkaline sodium hydrosulfite are placed in the cup, of which 1 cc. is slowly admitted into the chamber (1, 4). Stop-cock *b* is sealed and  $p_2$  is read at the 2 cc. mark.

*Determination of *c* Correction*—The *c* correction is determined with 1 drop of caprylic alcohol and 5 cc. of water. The water is extracted for 3 minutes in the evacuated chamber. This extracted air is expelled and 1.5 cc. of water are run up into the cup and discarded. 1 cc. of 1 N NaOH is admitted, the stop-cock sealed, and the extraction chamber evacuated and reextracted for 3 minutes. Reading  $p_1$  is taken at the 2 cc. mark. 1 cc. of alkaline sodium hydrosulfite is admitted as in the oxygen determination and the reading  $p_2$  taken.  $p_1 - p_2 = c$ .

*Calculations*—The results are calculated with the formula  $(p_1 - p_2 - c) \times f$ . The factor *f* is the same as used in the Van Slyke-Neill technique ((4) p. 543).

It is very important to clean the extraction chamber of the blood-reagent mixture thoroughly and to guard against carrying over any material in the use of the Hempel pipette, as it is noted that the presence of this solution in the extraction chamber, even in a small quantity, influences the results of the determination.

## EXPERIMENTAL

*Effect of Ether on Blood Oxygen Determinations by the Usual Van Slyke-Neill Technique*

The quantity of ether required for surgical anesthesia has been reported as varying from 86 to 200 mg. per 100 cc. of blood, with an average value of 100 to 150 mg.<sup>2</sup> In order to determine the extent that ether interfered with the Van Slyke-Neill (4) technique, dog blood was saturated with air in a Stadie rotator (13) and oxygen capacity determinations were made. Between 1 and 1.5 mg. of ether per cc. were then added, by means of a microburette, to the same blood samples *in vitro* and oxygen capacity was again determined in the usual manner.

*Results*—A series of five experiments was carried out which showed minimum and maximum deviations of 8 to 15 per cent in excess of the control values. The mean variation (14) for the group was  $+10.95 \pm 2.72$  per cent. These large variations make it evident that oxygen cannot be determined accurately by the usual technique when ether is present. A typical example is shown in Table I, Experiment 7.

*Application of Reextraction Principle to Oxygen Determinations*

Oxygen capacity determinations on blood were made and ether was added to the same sample of blood as described above. After the addition of ether, determinations were made according to the reextraction technique of Austin (2) and also by the modification of Fuss and Derra (3). These procedures vary from the usual technique of Van Slyke and Neill in that, after absorption of CO<sub>2</sub> by NaOH, the solutions are reextracted until checked readings are obtained (Austin) or a mean of the first four readings is taken (Fuss and Derra). Oxygen is then absorbed in the usual manner. The chamber is next evacuated and reextraction repeated until constant readings are obtained (Austin (2)).

<sup>2</sup> The concentration of ether per 100 cc. of blood as given by various writers is as follows: Gramén (7) 104 to 150 mg., White (8), Haggard (9), and Nicloux (10) 100 to 140 mg., Ronzoni (11) 130 to 180 mg., and van Leeuwen (12) 86 to 200 mg.

TABLE I  
*Comparison of Various Methods for Estimation of Blood Oxygen in Presence of Ether*

Experiment No.	Method	Ether added per cc. blood	Temperature	Manometer readings after addition of				O <sub>2</sub> ( <i>p</i> - <i>p</i> <sub>1</sub> - <i>c</i> )/	O <sub>2</sub> recovered according to			
				NaOH		Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>			Austin's principle	Fuss and Derra's technique	Authors' technique ( <i>p</i> <sub>2</sub> - <i>p</i> <sub>3</sub> - <i>c</i> )/	Authors' technique with K
				Before reex- trac- tion	After reex- trac- tion	mm. <i>p</i> <sub>1</sub>	mm. <i>p</i> <sub>2</sub>					
7*	Van Slyke-Neill	0	22.8	157.2		44.8		26.96				
		0	23.0	157.0		44.6		26.95				
	Van Slyke-Neill	1	23.0	168.9		44.7		29.80				
	Reextraction	1	23.0	168.4	174.0	50.3	72.5	(-9.8%)†	25.26	26.03		
					171.4		73.5					
					169.2		73.5					
					167.2							
					165.4							
					164.4							
					163.9							
					162.7							
		1	23.0	166.8	171.3	48.9	70.1		24.78	25.87		
					168.8		70.4					
					165.9		70.2					
					165.0				Average 25.02	Average 25.95		
					162.0				(-7.2%)†	(-3.7%)†		
					158.7							
					159.9							

7*- Con- tinued	Authors'†	1	22.8	150.9	156.7	44.9	46.5	.			26.8 (-0.58%)†	26.97 (+0.05%)†
10*	Van Slyke-Neill	0	24.0	159.1		46.8		26.82				
		0	22.7	156.5		44.6		26.86				
	Authors'†	1	23.5		158.5	46.7	48.2				26.75	26.91 (+0.28%)†
16*		1	23.5		158.5						26.75 (-0.33%)†	
	Van Slyke-Neill	0	25.6	142.0		43.0		23.49				
		0	25.7	142.0		43.0		23.48				
	Authors'†	1.5	24.9	138.5	139.5	41.0	41.6				23.42	
		1.5	23.4	136.2	137.0	39.0	40.0				23.42 (-0.25%)†	

\* Same blood used throughout each experiment.

† Per cent variation from control.

‡ Experiments 7 and 10, extracted gases stored over mercury in Hempel pipette while extraction chamber was cleaned; Experiment 16, extracted gases stored over glycerol-salt solution during cleaning.

*Results*—A series of seven experiments was carried out entailing twenty-five determinations of oxygen in the presence of ether, and from the results a correction factor was calculated according to Austin's formula ((1) p. 353; (2) p. 350).

$$K = \frac{O_2 - f(p_2 - p_1)}{f(p_4 - p_3)}$$

The value of factor  $K$  determined from the readings that checked on reextraction varied from 0.27 to 1.01 and gave a mean of 0.598. The recovery of oxygen was calculated with Austin's formula,  $O_2 = [p_2 - p_3 + K(p_4 - p_3)] \times f$ . The results with  $K = 0.598$  gave recoveries of maximum variations from the controls of  $-7.18$  and  $+11.7$  per cent and minimum variations of  $-1.14$  and  $+1.25$  per cent. The mean was  $-1.98 \pm 7.3$  per cent. Duplicate determinations in the presence of ether ranged from perfect checks to differences of as much as 7.5 per cent. A typical example is shown in Table I, Experiment 7.

Fuss and Derra (3), in their modification of Austin's procedure, used the mean of the first four reextractions to calculate the factor  $K$ . The value of  $K$  determined in this way varied from 0.26 to 0.7, giving a mean of 0.43. In this group of experiments  $K = 0.43$  gave oxygen values with maximum variations of  $-3.7$  and  $+6.5$  per cent and minimum variations of  $-1.18$  and  $+0.83$  per cent. The mean variation from the control was  $-0.93 \pm 4.07$  per cent. Duplicate determination differences ranged from 0.7 to 2.9 per cent.

The seven experiments by either method of calculation showed a recovery in excess in four instances and a deficit in the remaining three. The mean variations of the two methods of calculation indicate that the maximum error may be approximately 10 per cent when manometer readings are used that check on reextraction and 5 per cent when the mean of the first four reextractions is used (Fuss and Derra). Both of these methods were tedious and time-consuming. Frequently in order to obtain checks on reextraction eight or ten shakings, requiring as much as 30 minutes, were necessary to obtain manometer readings within a few tenths of a mm. In addition to this difficulty it was apparent that several variables were present, making estimations even more unreliable than the average of the results would indicate.

*Use of Hempel Pipette for Oxygen Determinations in Presence of Ether*

The foregoing experiments demonstrate that changes in temperature, or in the reaction of the solutions in the chamber, produced variations in the vapor pressure exerted by the ether, which acted quite independently of the gases being measured. Since most of the ether<sup>3</sup> is dissolved in the blood-reagent mixture, it seemed that the removal of this material from the apparatus before the actual manometric measurement of gases was made would simplify the procedure considerably. Two devices for accomplishing this removal have already been described for use in other procedures with the Van Slyke apparatus: the Harington-Van Slyke extraction chamber (16) and the modified Hempel pipette (5). The experiments recorded here were made with the Hempel pipette because of its availability to laboratories already having the ordinary Van Slyke apparatus.

The oxygen capacity was determined on dog blood by the usual Van Slyke-Neill technique. Ether was added as previously (1 to 1.5 mg. per cc.) to the same sample *in vitro* and determinations were made with the use of the Hempel pipette as described in the procedure.

*Use of Mercury in Hempel Pipette*—Thirteen experiments were carried out entailing twenty-four determinations of oxygen in the presence of ether. The maximum variation from the control oxygen capacity was  $-0.8$  per cent and the minimum was  $-0.02$

<sup>3</sup> The distribution ratio, *i.e.* quantity in solution to quantity in air, as given by Shaffer and Ronzoni (15) for the systems of water-air and blood-air at room temperatures, is as follows:

Temperature	Water-air system	Blood-air system
°C.		
20	39.0	34.5
25	29.0	26.0
30	21.0	20.3

Assuming that the distribution ratio in the extraction chamber between the liquid and gas phases lies in the vicinity of the above values, there will be, varying with the temperature, between 20/21 and 39/40 of the total ether in solution with the blood-reagent mixture. Therefore, the removal of the blood-reagent solution from the apparatus would eliminate practically all the interfering ether.

per cent. The mean variation for the entire group was  $-0.31 \pm 0.39$  per cent. Duplicate determination differences in the presence of ether were all less than 1 per cent, except in two instances in which they were less than 2 per cent. The average duplicate difference for the entire group was 0.68 per cent. In the thirteen experiments the recoveries in eleven instances were less than the controls and in excess in the remaining two. Readings on reextraction were easily checked, usually with the first two attempts.

The difference between the  $p$  readings of the Hempel pipette technique and the  $p$  readings of the controls (see Table I) were very small and frequently the readings were almost alike. There was a close parallelism of difference between respective manometer readings of the control and the ether-containing bloods.

In this same group of experiments a correction factor,  $K$ , was calculated after the manner of Austin (2). The correction factor obtained varied from 0.215 to 1.04 and gave a mean value of 0.683. Calculations based on this factor gave maximum errors of recovery of  $+0.53$  and  $-0.42$  per cent and minimum errors of  $-0.04$  and  $+0.03$  per cent. The mean variation from the controls was  $0 \pm 0.25$  per cent. The differences in duplicate determinations in the presence of ether were less than 1 per cent, except in three experiments in which they were less than 2 per cent. The average difference in duplicates was 0.58 per cent. In eleven experiments the recoveries were in excess in six instances and below the average in the remaining five cases.

The difference between the  $p$  readings of the Hempel pipette technique and the  $p$  readings of the control bears out the theoretical assumption that most of the ether is removed with the discarded blood-reagent solution. The small quantity of ether remaining does not appear to interfere appreciably with the determination as shown by the accuracy of the results, the parallelism of difference between the respective manometer readings of the control and the ether-containing bloods, and the ease with which checked readings are obtained following reextraction. For routine purposes one could rely upon the first reextraction, since there is rarely a difference greater than a few tenths of a mm. between the first reading and the checks.

The use of Austin's principle with the Hempel pipette increases the accuracy of the method to a maximum variation from the

control of about  $+0.5$  per cent, whereas the Hempel pipette alone showed a variation from the control of  $-0.81$  per cent. Considering the labor of determining the  $K$  factor and the additional extractions and calculations, however, it would seem that the added inconvenience is unwarranted for most experimental purposes.

*Use of an Air-Free Glycerol-Salt Solution in the Hempel Pipette*—The use of mercury in the Hempel pipette presents two distinct disadvantages: its weight, which makes the Hempel pipette quite difficult to handle, and its inability to absorb the portion of ether transferred along with the extracted gases from the extraction apparatus. The elimination of either one of these disadvantages would be a decided improvement. So with this in mind several solutions were tried as a substitute for mercury. A saturated solution of calcium chloride gave very variable results. Concentrated sulfuric acid, which readily absorbs ether, was found to form a precipitate with the blood-reagent mixture which made it impossible to manipulate the Hempel pipette. Both ideas were abandoned as impractical.

In a personal communication to us Dr. Van Slyke suggested that the substitution of a solution of equal parts of glycerol and saturated sodium chloride for mercury would work as efficiently and possibly in addition absorb the remaining interfering ether. Since this solution has a very low coefficient of solubility for gases in general, the loss of oxygen over the small interval of time during the determination would be negligible and, according to the distribution ratio<sup>3</sup> of ether between a gaseous and a liquid phase, practically all of the ether should be removed. Accordingly the Hempel pipette was filled with an air-free glycerol-salt solution which was protected from the air by a layer of paraffin oil. Air-free solution was used, so that if a small amount of solution was accidentally carried over into the Van Slyke-Neill apparatus there would not be any dissolved air present to influence the determination on reextraction. The plan and procedure of experiment as outlined above were pursued, except that the Hempel pipette was filled with an air-free glycerol-salt solution.

In twelve experiments, twenty-six determinations of oxygen in the presence of ether were made. The maximum variations from the controls were  $+0.73$  and  $-0.75$  per cent and the minimum  $-0.09$  per cent. The mean variation for the entire group was



$-0.21 \pm 0.58$  per cent. In ten of these experiments on which duplicate determinations in the presence of ether were made, there were the following differences. In seven the range was from 0 to 1 per cent; in two others the range was from 1 to 2 per cent; and one differed by 2.2 per cent. The average difference for the whole group was 0.8 per cent. In the twelve experiments, recoveries were less than the controls in eight instances and in excess in the remaining four cases. Checks on reextraction were usually obtained on the first two attempts.

The employment of the glycerol-salt solution appears to absorb all or a major portion of the ether remaining in extracted gases while in storage in the Hempel pipette. This is indicated by the very small difference between the  $p_4$  reading (following reextraction) (see Table I) and the  $p_3$  reading (following the addition of  $\text{Na}_2\text{S}_2\text{O}_4$ ). The difference was rarely over 1 mm., which is practically the same as that obtained following reextraction at this point in the usual Van Slyke-Neill technique. Further proof of absorption of the ether was noted by the recovery of oxygen in slight excess in about one-third of the cases when  $p_4$  was used in the calculations. In these instances it was impossible to calculate a correction factor. There was with the glycerol-salt solution a slight tendency to obtain more variable results and wider differences in duplicate determinations. This is particularly true in cases of accidental passage of a quantity of glycerol-salt solution from the Hempel pipette to the extraction chamber. Since there may be quite a quantity of ether dissolved in the solution after usage, it is advisable to use great care in transferring the gases to prevent the carrying over of this solution into the extraction chamber. The authors have used an air-free glycerol-salt solution for as many as ten determinations with satisfactory results, though no experiments were carried out to determine the possible life of such a solution. In view of the negligible difference in accuracy it is advised that the more flexible glycerol-salt solution is satisfactory for most experimental procedures. However, for the utmost accuracy, mercury is recommended with the employment of a correction factor.

#### SUMMARY

1. Data are presented to show that  $\text{O}_2$  determinations by the Van Slyke-Neill technique in the presence of ether may be as much as 15 per cent in excess of the correct value.

2. A modification of the Van Slyke-Neill method is presented and described for determining  $O_2$  in blood in the presence of ethyl ether with an error of less than 1 per cent of true values.

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# PURIFICATION OF THE DEPRESSOR COLLOID OF URINE (CALLICREIN)

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The presence of a depressor fraction in normal urine was first noted in 1903 (1). 17 years later the fraction was shown to be non-dialyzable (2). The possible significance of these isolated observations was not realized until Frey (3) made a thorough investigation of the chemical and physiologic properties of this urine fraction. While his observations hardly justify his characterization of the substance as a "circulatory hormone," they and more recent observations by Elliot and Nuzum (4) nevertheless indicate that the fraction is worth further study. It is probable that hypoglycemic properties and calorigenic effects (5, 6) reported in the literature, are attributable to contaminating impurities. Crude prolan<sup>1</sup> and the tumor resistance factor of urine (7) are contaminated with the depressor fraction. The difficulty of removing the depressor colloid from the urine colloids mentioned above, which possess well defined physiologic properties, is an added incentive to a study of its properties.

In this paper are described the procedures by which the depressor colloid of the urine has been obtained in a purer form than that described by Kraut *et al.* (8). Before attempting this study, the conditions affecting the stability of the crude depressor colloid

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<sup>1</sup> Unpublished data from this laboratory. A crude 80 per cent alcohol precipitate of concentrated urine of pregnancy (method of Zondek) contained 6 Elliot units per mg. A sulfosalicylic acid precipitate (method of Dickens) after one alcohol precipitation assayed 385 Elliot units per mg. of N.

were first established, so that procedures which would bring about partial inactivation could be avoided (9). The following innovations and departures from the procedures of Kraut *et al.* have been introduced. (a) The pH never exceeds the limits of stability of the crude product. (b) Impurities which cannot be removed by centrifugation are removed by the Berkefeld filter. (c) Acid adsorption is performed by utilizing the acid-insoluble colloids of the urine. (d) Fractional alcohol precipitation is effected by means of electrolytes. (e) Zinc hydroxide is used as an adsorbing agent. (f) Concentration *in vacuo* with subsequent dialysis replaces the cumbersome uranium precipitation.

#### EXPERIMENTAL

*Physiologic Standardization*—The physiologic activity of all the preparations described in this paper was evaluated in Elliot units (4). The relation between the Elliot unit and the Frey unit is unfortunately not clearly established, 1 Frey unit equaling 10 to 28 Elliot units, depending upon the age of the individuals whose urine is taken for the Frey standard.

*Preliminary Purification*—10 liter batches of urine, collected either from males or females and preserved with toluene, are concentrated *in vacuo* below 45° to 2 liters or less. 20 gm. of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  dissolved in the minimum amount of water are added per batch and sufficient aqueous ammonia to bring the pH alkaline to brom-thymol blue and acid to thymol blue. The heavy precipitate is removed by centrifugation and the supernatant liquid dialyzed in collodion or parchment membranes for 48 hours against water saturated with chloroform. It has been found convenient to use La France condoms for this purpose. These membranes are readily preserved in water saturated with chloroform and may be used over and over again. The membrane contents are concentrated *in vacuo* below 45° to 300 cc. volume, again subjected to dialysis, centrifuged to remove the inactive precipitate which has formed, reconcentrated to a 100 cc. volume, and filtered through a Berkefeld filter, the latter step removing more inactive material. Filtrates prepared in this manner contain from 20 to 500 units per mg. of N, with an occasional degree of purity representing 1000 units per mg. of N.

*Acid Precipitation*—In the method of Frey, the product dialyzed

to remove electrolytes after elution from the uranium precipitate is adsorbed on benzoic acid, which later is removed by alcohol-ether extraction. Dilute alkali is necessary to effect solution of the precipitate. It occurred to us that the benzoic acid procedure was not properly an adsorption, but merely served to bring about a degree of acidity necessary to precipitate the natural urine acid-insoluble compounds which themselves adsorbed the substance. It was found that most preparations, which had gone through our preliminary purification procedure, became opalescent on the gradual addition of mineral acid, until a precipitate formed at pH 4.2 to 4.5. This precipitate was insoluble on further addition of acid and, if taken up in dilute alkali solution, contained active material having a lower nitrogen content than that of the original solution. Per unit of nitrogen, the activity might be increased several hundred per cent. If the precipitate was dried, considerable loss of potency resulted. The pH at which the precipitate is thrown down, pH 4.2, was found slowly to inactivate the substance.

In order to ascertain whether the benzoic acid precipitation procedure served merely to bring about the required degree of acidity for precipitation of the urinary substances or actually adsorbed active material, several batches of material were subjected first to an acid precipitation with hydrochloric acid or acetic acid and then to a benzoic acid precipitation. It was found that considerable material was adsorbed by the benzoic acid, that the active material so adsorbed was less pure than that adsorbed by the urinary substance, and that it was acid-soluble. In some instances the dispersion conditions are such that the addition of mineral acid results in a cloudiness. The acid-insoluble colloid cannot be removed by centrifugation. In an example of this kind benzoic acid was found to throw down the acid-insoluble colloid with some acid-soluble material and effect a purification (see Table I).

The active material which is not removed by an acid precipitation and which may constitute over three-fourths of the original activity may be separated by an 80 per cent alcohol precipitation. The product so obtained is always acid-soluble and may be as pure or purer than the material which is acid-insoluble.

*Alcohol Purification*—We observed that the alcohol precipitation of active material is dependent upon the electrolyte concentra-

tion and that our purest preparations were precipitated only from 75 per cent alcohol when saturated with NaCl. Less pure preparations required less salt.

Crude preparations can always be purified by a 75 per cent alcohol concentration precipitation. Often inactive material is first removed at 50 per cent alcohol concentration, effecting a still greater purification. In very crude preparations some potency is adsorbed by the 50 per cent alcohol precipitate. Inactive material not precipitated in the first 50 per cent alcohol precipitation may be rendered insoluble at this concentration in subsequent alcohol

TABLE I  
*Effect of Successive Acid (HCl) and Benzoic Acid Precipitations*

Purification procedures before pptn.	Potency, units per mg. N			
	Original potency	HCl ppt.	Benzoic acid ppt.*	Final filtrate
Uranium ppt. of Frey.....	135	1050	570	
Concentration <i>in vacuo</i> , dialysis,	480	3120	150	150
Berkefeld filtration, and 78% alcohol pptn.	285	No ppt.	2100	40
Concentration <i>in vacuo</i> , dialysis, 78% alcohol pptn.	580	No ppt.	2600	

\* 3 gm. of benzoic acid in a saturated alcohol solution added to 60 cc. of solution.

precipitations. The results given in Table II are the averages of a series of five, three, and two determinations respectively.

The purest preparations were obtained by fractional precipitation from alcohol at 75 per cent concentration. Relatively inactive material was removed by the addition of 0.5 to 1 per cent NaCl. Highly potent material then separated on standing or on the addition of more salt. Details of such purifications are given in Table III.

Frey and his coworkers abandoned the use of fractional alcohol precipitation because they found it affected the stability of purer preparations. Beyond a certain degree of purification all our preparations have been unstable, whether or not alcohol is used. Since fractional alcohol precipitation is the most effective method of purification found, the state of purity of the resulting product and not the alcohol itself may be responsible for the loss in potency.

*Zinc Hydroxide Adsorption*—Material approximating 1000 units per mg. of N is usually not greatly adsorbed by  $\text{Zn}(\text{OH})_2$  at a pH

TABLE II  
*Examples of Purification by Alcohol Precipitation*

Starting material	EtOH-NaCl concentration	Potency, units per mg. N		
		Before pptn.	Ppt.	Filtrate
Preliminary purification; filtrate from 50% EtOH pptn.	75% EtOH; natural electrolytes	60	335	
Preliminary purification; filtrate from acid pptn.	80% EtOH; 0.2% NaCl; natural electrolytes	920	3100	
Preliminary purification; 75% EtOH pptn.	47% EtOH; 1.6% NaCl	350	80	450

TABLE III  
*Effect of Alcohol Precipitation Following  $\text{Zn}(\text{OH})_2$  Adsorption*  
The values are expressed in units per mg. of N.

Starting material		First ppt.		Second ppt.		
Filtrate, 1 zinc adsorption	1200	75% EtOH 2% NaCl	4,000			
Filtrate, 3 zinc adsorptions	400	72% EtOH 1.4% NaCl	170	78% EtOH 1.2% NaCl		1,300
$\text{NaHCO}_3$ extract, 3rd zinc adsorption	6700	75% EtOH 0.4% NaCl	None	75% EtOH 0.8% NaCl		12,000*
Filtrate, 3 zinc adsorptions	900	75% EtOH 0.7% NaCl	1,300*			
$\text{NaHCO}_3$ extract, 4th zinc adsorption	8100	75% EtOH 0.8% NaCl	None	75% EtOH Saturated NaCl		85,000*
Filtrate, 2 zinc adsorptions	1900	75% EtOH Trace, NaCl	5,000	75% EtOH 2% NaCl		None
$\text{NaHCO}_3$ extract, 3rd zinc adsorption	940	75% EtOH 1.2% NaCl	10,400*			

\* Unstable product.

alkaline to phenolphthalein, while considerable inactive material may be removed. If the starting material is at pH 7.0, the filtrate



after adsorption will be alkaline to phenolphthalein, presumably due to a double decomposition reaction between the sodium salts of the inactive materials and the  $\text{Zn}(\text{OH})_2$ , and will contain the active material. If the resulting solution be again taken to neutrality and the process repeated several times, no change in pH will result and the active material will be adsorbed. In that case or in those cases in which the active material is adsorbed at an alkaline pH, the active material can be reliberated by extracting with  $\text{NaHCO}_3$  solution, a process which again results in further elimination of nitrogen (see Table IV).

TABLE IV  
*Effect of  $\text{Zn}(\text{OH})_2$  Adsorption*

Product after $\text{Zn}(\text{OH})_2$ adsorption	Reaction during adsorption	Potency, units per mg. N	
		Before adsorption	After adsorption
	<i>pH</i>		
1. Filtrate, 1 adsorption	> 8.0	800	1,100
"    3 adsorptions	> 8.0	1100	3,000
$\text{NaHCO}_3$ extract, 4th adsorption	< 8.0	3000	8,100
2. Filtrate, 2 adsorptions	> 8.0	1400	1,900
$\text{NaHCO}_3$ extract, 3rd adsorption	7.4	1900	20,000*
3. Filtrate, 3 adsorptions	9.0, 8.2, 9.2	490	440
$\text{NaHCO}_3$ extract, 1st adsorption	9.0	440	710
4. Filtrate, 2 adsorptions	> 8.0	1700	1,400
$\text{NaHCO}_3$ extract, 3rd adsorption	7.4	1400	3,100

\* Unstable product.

The zinc hydroxide suspensions were prepared by adding N NaOH to a 2 per cent zinc sulfate solution until the reaction was alkaline to phenolphthalein. The precipitate was separated by centrifugation and washed five times with water. About 300 mg. of  $\text{Zn}(\text{OH})_2$  were used for 100,000 units of material in a volume of about 100 cc. The adsorption time ranged from 5 to 30 minutes. Two 1 gm. portions of sodium bicarbonate in 30 cc. volume were used for elution.

*Stability*—The purest and least stable preparations were obtained by alcohol precipitation in the presence of sodium chloride of solutions which had been subjected to a  $\text{Zn}(\text{OH})_2$  adsorption.

In some instances almost complete inactivation (over 90 per cent) occurred in 5 days. This occurred whether the alcohol precipitate was allowed to dry spontaneously or was dissolved in water. A lesser degree of loss of potency was observed for material adsorbed by  $\text{Zn}(\text{OH})_2$  and reliberated with  $\text{NaHCO}_3$ . In these preparations a certain loss of potency occurred during the first 10 days, without further loss.

The effect of such factors as precipitation by acetone (80 per cent), precipitation by alcohol (75 per cent), drying over  $\text{P}_2\text{O}_5$ , solution in water, temperature (room temperature *versus*  $0^\circ$ ), and solution in 50 per cent glycerol were studied simultaneously for the same sample. A product which had been subjected to three zinc hydroxide adsorptions and assayed 2000 units per mg. of N was used as the starting material. The results showed that stability already affected by  $\text{Zn}(\text{OH})_2$  adsorptions (45 per cent loss in potency) was further decreased by alcohol or acetone precipitation (90 per cent loss in potency) with concomitant purification (removal of 41 per cent nitrogen). The dried samples lost less potency than those kept in aqueous or glycerol solution.

*Analytical Data for Nitrogen and Phosphorus*—The nitrogen content in terms of organic matter (loss on ashing) of products obtained by us varied from 12.7 to 1.5 per cent. The purest product contained 3.4 per cent N. Three acid-insoluble products, assaying about 150 units per mg., contained 8.7, 8.8, and 9.9 per cent N. Two acid-soluble products of the same potency gave on analysis 5.1 and 7.0 per cent N. Kraut's lowest phosphorus value was 0.00006 mg. of P per 1 Frey unit. Our lowest phosphorus-containing sample assayed 180 Elliot units for the same amount of phosphorus, which approximated the content of 1 mg. of organic matter (loss on ashing). 0.25 per cent organic phosphorus was, however, found in a sample which assayed 770 units per mg. Kraut *et al.* (8) were unable to detect any characteristic chemical group in preparations corresponding to our 100 units per mg. material. Since our purest preparations had 20 times the above activity, we decided that analytical studies of crude preparations could lead to little information in regard to the chemical nature of the pure compound or compounds possessing the active groups. None has therefore been made. The available material of our purest prod-

ucts sufficed only for physiologic standardization, microdetermination of nitrogen and phosphorus, and ash determination.

#### DISCUSSION

The purest stabile products obtained by Kraut *et al.* (8) entailed the following steps in preparation: uranium precipitation, elution by phosphate, dialysis, benzoic acid precipitation, and charcoal adsorption. The same degree of purification was obtained by us in the following procedures: concentration *in vacuo*, dialysis, acid precipitation, and fractional alcohol precipitation. 0.05 mg. of such a preparation neutralizes the pressor effect of 0.01 mg. of adrenalin administered intravenously to the amygalized rabbit. Beyond the preliminary purification directions, neither Frey and his coworkers nor ourselves can describe a process which will give consistent results. It is necessary to determine the physiologic activity after each procedure.

By means of selective adsorption at various reactions on zinc hydroxide of either active material or impurities, followed by fractional alcohol precipitation, a further purification (usually 2- to 5-fold) may be effected. Unstable products are the rule. For this reason it was necessary to arrange for biologic standardization on the day of preparation in these experiments. Occasionally a product which assayed 400 Elliot units per mg. (twice the potency described above) and which was stabile in solution was obtained. The purest product, which rapidly lost its activity, assayed 2900 Elliot units per mg. when first tested. With but one exception the products have been amorphous. The exception, a well defined crystalline precipitate from alcohol, contained 58 per cent ash combined mostly as bicarbonate. Whether the crystalline aggregate was a definite chemical entity, a double salt of the depressor colloid with bicarbonate, or whether the activity was merely adsorbed remains to be investigated on duplication of the crystal formation, which has not again been attained. A similar situation exists at present in regard to prolan (10).

It would appear that information regarding the nature of the inactivation of the purer products is necessary before they can be prepared consistently in quantities large enough for chemical and physiologic studies. Regardless of the instability of the highly

purified product, one is impressed by the high degree of physiologic activity, rivaling as it does that of adrenalin, with which the depressor colloid is most properly compared.

#### SUMMARY

1. A separation of the depressor colloid of urine in stabile form is described. The procedures entail concentration *in vacuo*, dialysis, acid precipitation, and fractional alcohol precipitation.

2. A degree of purification not heretofore described is attained by subjecting the purified stabile preparation to selective adsorption on zinc hydroxide, with subsequent fractional alcohol precipitation. The products are usually unstable.

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## A QUALITATIVE TEST FOR ENZYMES OF THE TRYPSIN AND PAPAIN TYPES

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Carmine fibrin (1)<sup>1</sup> has been used as a qualitative reagent for pepsin for many years, but cannot be used in tests for trypsin or other proteases which act at neutrality or at an alkaline pH. In place of carmine fibrin, Congo red fibrin (2) and spirit blue (3) fibrin have been advocated for the detection of trypsin. We have found both of these unsatisfactory. Recently we have tried hide powder containing barium sulfate precipitated within its pores. When this material is acted upon by proteases, the barium sulfate is liberated and goes into suspension upon shaking. The reagent can be employed for the detection of all proteases thus far tested which attack the high molecular proteins, but is not suitable for pepsin. The reagent is easy to prepare and inexpensive. Hide powder can be purchased from any chemical supply house.<sup>2</sup>

With the hide powder-barium sulfate a positive test is given by 1 mg. of Fairchild's 1:3000 trypsin at pH 7.0 at room temperature

<sup>1</sup> We have observed that carmine fibrin prepared with some samples of carmine is not attacked by pepsin. Apparently some heavy metal was present, for carmine solutions saturated with hydrogen sulfide served for the preparation of excellent carmine fibrin. Such carmine fibrin gave a positive test with 1 mg. of 1:3000 Park, Davis pepsin in 2 minutes at room temperature.

<sup>2</sup> Our hide powder is manufactured by the Standard Manufacturing Company of Ridgway, Pennsylvania. The company states that it is prepared by liming hide as is done in a tannery. The hide is then delimed and brought to pH 5.0 to 5.4. It is then dried and ground to a powder. It is not ground to pass through any special size sieve, but to conform with the specifications of the American Leather Chemists Association and the International Association of Leather Trades Chemists.

in 1 minute. 1 mg. of Park, Davis 1:3000 pepsin at pH 2.0 gave practically no test for 4 hours, but large quantities of pepsin gave a test rapidly. Fig tree protease,<sup>3</sup> taka-diastrase preparation, papain-cysteine,<sup>4</sup> and bromelin-cysteine gave positive tests at pH 5.0 very rapidly. Extract of green malt and a commercial saccharase preparation gave positive tests at room temperature at pH 4.1 only after about 1 hour. As little as 0.001 mg. of Fairchild's trypsin gave a positive though faint test after incubation with the hide powder at 37° overnight. For some unexplained reason the rate of digestion decreases very rapidly with small amounts of trypsin.

The test is carried out as follows: Place about 0.3 gm. of dry hide powder-barium sulfate in two 17 × 155 mm. test-tubes and cover with a little distilled water. After 5 or 10 minutes add the desired buffer solution and enough water to make a total volume of 5 to 10 cc. Now add about 1 cc. of clear enzyme solution to one of the tubes and shake both tubes for 30 to 60 seconds. Allow the powder to settle and observe whether the tube containing the enzyme shows turbidity. It will help to make a comparison with the blank. If there is no turbidity to be seen, allow some time to elapse and later shake again and make other observations. Sometimes enzyme solutions themselves become turbid upon being added to buffers, or simply upon standing. It is therefore advisable to have a third tube containing only enzyme, buffer, and water. It is, of course, necessary to employ enzyme solutions which are water-clear. In testing for bromelin we used fresh pineapple juice. This was difficult to filter unless diluted first with 2 volumes of water.

The control tube will not give off any barium sulfate at pH 5.0 or 7.0 upon incubation overnight at 37°. However, barium sulfate will be released if the material is boiled, or if the solid is pounded with a glass rod. In the presence of 0.1 N hydrochloric acid barium sulfate is given off slowly.

The reagent is prepared as follows: Place 1000 cc. of 5 per cent barium chloride in a 2 liter beaker and bring to boiling. Add 100 gm. of hide powder and at once turn off the flame heating the

<sup>3</sup> This was kindly furnished to us by Professor B. H. Robbins of Vanderbilt University.

<sup>4</sup> Boehringer papain, 1:350, was employed.

beaker. Stir for 10 minutes. Now pour the material upon a cheese-cloth sack resting in a filter funnel and squeeze out the excess of liquid. Then dump the moist hide powder into a 2 liter beaker containing about 1000 cc. of 10 per cent ammonium sulfate and stir for 5 to 10 minutes. Pour the material into the cheese-cloth sack and press out the liquid. Transfer the moist hide powder to the beaker again and stir with 1000 cc. of distilled water. Repeat this washing process about nine times. Finally, spread the hide powder-barium sulfate out in a thin layer upon towels or several layers of cheese-cloth. Break up all lumps. Allow to remain until perfectly dry. The material should not be employed as a reagent until it has dried thoroughly.

#### SUMMARY

A method is given for preparing hide powder-barium sulfate. The employment of this material as a qualitative test for trypsin, papain, and other proteases is described.

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# THE CHEMICAL DETERMINATION OF MINUTE QUANTITIES OF VITAMIN C

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The microestimation of various enzymes and other substances of biological significance, by use of the apparatus and technique devised by Linderstrøm-Lang and Holter, has made possible histological-chemical studies of various plant and animal tissues. This technique permits chemical determinations on single microtome sections of tissue, or other biological units of similar magnitude (1, 2). It is the purpose of the present investigation to extend the scope of this technique to include the estimation of vitamin C.

There have been a number of attempts to determine the histological distribution of vitamin C by the use of the silver nitrate staining reaction (3-6). The black deposit of reduced silver is taken as an indication of the presence of the vitamin. This method has been useful in many instances, but at best it is only semiquantitative, and in some cases it may not be specific for vitamin C. Harris and Ray (7) have pointed out that the medulla of ox adrenal does not stain with silver, though the cortex does; still the medulla contains vitamin C in almost the same order of concentration as the cortex. The difficulties with this indirect staining method are obviated by the direct procedure to be described.

Birch, Harris, and Ray (8) have devised a microchemical method for the determination of vitamin C, based on the titration of the vitamin with 2,6-dichlorophenol indophenol, according to the method introduced by Tillmans and his associates (9). These authors have placed the unknown vitamin solution in a burette graduated in 10 c.mm., and titrated it into a tube containing 50 c.mm. of the dye (about 0.01 M). The procedure to be described is a refinement of this method, allowing about one-fiftieth the

amount of the vitamin to be determined without loss of accuracy. Thus, in the procedure herein presented, titration of vitamin C can be reproduced to  $\pm 0.20$  c.mm. (equivalent to about 0.0001 mg. of pure vitamin) of practically saturated 2,6-dichlorophenol indophenol.

#### EXPERIMENTAL

The extraction of the vitamin C from tissues and the preparation and standardization of the dye are essentially the same as described by Bessey and King (10). The titration was conducted according to the Linderstrøm-Lang-Holter technique, with the special re-reaction vessels, pipettes, burette, and magnetic stirring (1, 2).

The microburette employed was of the Type 2 variety, in which the mercury does not come into contact with the standard solution. The smallest divisions on the burette are 0.20 c.mm., but readings are taken to 0.02 c.mm. The dye, rather than the vitamin C solution, was placed in the burette.

Because of the interference of trichloroacetic acid solutions in the titration (10), a 9 per cent solution of acetic acid was used as the tissue extractant throughout. All titrations were carried out in the presence of 50 c.mm.<sup>1</sup> of 9 per cent acetic acid, and the end-point was determined by comparison with a color standard composed of 50 c.mm. of dilute rose Bengal solution placed in one of the reaction vessels. The rose Bengal solution employed was the most dilute solution, having a distinct pink tinge when placed in a reaction tube and compared with a similar vessel containing water (about 1 part per million was the concentration used).

It was observed that the titratable vitamin C contained in the stoppered reaction vessels gradually diminished, probably owing to reaction with the oxygen in the air over the liquid. This difficulty could be overcome when the stoppered vessels were immersed in a salt-ice freezing mixture, as demonstrated in Table I.

The application of this technique to the determination of vitamin C in microtome sections of tissue is shown in Table II. Liver was chosen, since it has a rather homogeneous histological structure, so that with serial sections there should not be a radical difference between the vitamin content of individual slices. Rab-

<sup>1</sup> Measured with the so called hand pipette (1, 2).

bit liver, used immediately after the animal was killed, was frozen stiff, and a cylinder of tissue removed with a cork borer of 4.5 mm. diameter. The tissue was placed on a freezing microtome of

TABLE I

*Titration of 6.95 C.Mm.\* of Pure Vitamin C Solution (0.0001 Mg. per C.Mm.) in Presence of 50 C.Mm. of 9 Per Cent Acetic Acid*

Titrated at once	Titrated after 3 hrs. in freezing mixture
<i>c.mm. dye</i>	<i>c.mm. dye</i>
2.04	2.10
2.16	2.00
2.20	2.20
2.04	2.12
	2.00
Average . . . . . 2.11	2.08

\* A Type 1 pipette with microscopic attachment was used to measure the exact volumes (1, 2).

TABLE II

*Vitamin C Content of Microtome Sections of Rabbit Liver*

Order in which sections were cut	Thickness and No. of sections	Titrated at once	Titrated after 1 hr.
		<i>c.mm. dye</i>	<i>c.mm. dye</i>
1	1 of 40 $\mu$	1.12	
2	2 of 20 $\mu$	1.20	
3	2 of 20 $\mu$		1.20
4	1 of 40 $\mu$	1.16	
5	1 of 40 $\mu$		1.18
6	2 of 20 $\mu$	1.24	
7	2 of 20 $\mu$		1.24
8	1 of 40 $\mu$		1.28
9	1 of 40 $\mu$	1.20	
10	2 of 20 $\mu$		1.22

Titration values of 50 c.mm. of 9 per cent acetic acid without sections were 0.66, 0.72, 0.76, 0.68, average 0.70 c.mm. (this amount of dye was required to give the liquid the same color as the standard).

the rotary type and single sections of 40  $\mu$  thick, or 2 of 20  $\mu$ , were cut off and placed in separate reaction vessels containing 50 c.mm. of 9 per cent acetic acid. The tubes were stoppered and placed

in a freezing mixture at once. Some were titrated immediately and the others after 1 hour.

It will be seen from Table II that the vitamin is completely extracted almost at once, since the titration values do not change appreciably after standing. Furthermore, practically the same value is obtained with two sections 20  $\mu$  thick as with one of 40  $\mu$ , thus indicating no flaw in sectioning technique and no difference due to the increased surface in the case of the thinner sections.

#### SUMMARY

A method for the estimation of vitamin C has been described, which is reproducible to  $\pm 0.0001$  mg. It is suitable for determination of the vitamin C content of extremely small amounts of material, including microtome sections of tissue.

The author wishes to express his gratitude to Professor C. G. King of the University of Pittsburgh for the gift of the pure vitamin C and the dye used, and to Doctor G. R. Biskind of the Pathology Department of the Mount Zion Hospital for his help in this work.

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## THE MUCILAGE FROM PSYLLIUM SEED, *PLANTAGO PSYLLIUM*, L.

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The seed coats of psyllium seed consist of cells filled with mucilage (1). While chemical analyses of the seed, seed coats, and the mucilage have been made (2), nothing is known of the exact composition of the mucilage.

### EXPERIMENTAL

*Preparation of Mucilage*—White, imported psyllium seeds were mixed with from 10 to 30 times their weight of water and allowed to stand for between 10 and 30 hours. The solution was then pressed through cloth and the mucilage was precipitated by addition of 3 volumes of 95 per cent ethanol. In some cases the extraction was repeated a second and third time. In other cases the mucilage was isolated by extracting the seed for an hour with hot water. The yield of mucilage obtained by thorough extraction was approximately 20 per cent of the weight of the seed used.

The mucilage is a white, fibrous material, which is insoluble in alcohol. It swells with water to give a thick solution. This solution is neutral to litmus. It does not reduce Fehling's solution and gives no test for starch.

*Composition of Mucilage*—The results of the analysis of the mucilage are given in Table I. The data prove that it is a mixture. Its composition depends on the procedure followed in its preparation. The use of a small volume of water for a short time and the application of little force in pressing the solution through the cloth lead to production of small amounts of the mucilage with high ash and high uronic acid content but low pentosan content. On the other hand, the use of more water, longer extraction, and greater pressure in forcing the solution through the cloth

leads to production of larger amounts of mucilage with lower ash and lower uronic acid content but higher pentosan content. Second and third extractions of the seed with additional water yield a larger total amount of mucilage. These later crops contain, however, successively lower ash and uronic acid content but higher pentosan content. It thus appears that the polyuronides with higher uronic acid content dissolve most readily.

TABLE I

*Composition (in Per Cent) of Mucilage from Psyllium Seed under Different Conditions*

Crop No.	CO <sub>2</sub>	Uronic anhydride	Ash	Pentosan	X body	Mucilage obtained	Method of preparation
1	3.4	13.6	3.94	78	2.34	6.5	12 volumes water for 24 hrs.; little force used in pressing mucilage through
2	2.09	8.36	1.33	88.5		15	25 volumes water for 24 hrs.; much force used in pressing mucilage through
3-A	3.06	12.24	3.81	77		3	25 volumes water for 6 hrs.; little force used
3-B	1.22	4.88	1.22	90	1.54	14	10 volumes water added to residue from Crop 3-A; let stand 18 hrs.; much force used
3-C	0.92	3.68	0.80	91		3	5 volumes water added to residue from Crop 3-B; let stand 6 hrs.; much force used
4	1.8	7.2	1.00	90		90	Mucilage prepared from seed coats; 80 volumes water used for 18 hrs.

Attempts to separate the mucilage into a definite polyuronide and a pentosan free of uronic acid were unsuccessful. All of the pentosan seems to be combined with a uronic acid. This point has not, however, been thoroughly investigated. Future work may prove that a part of the pentosan is in the free condition.

Qualitative tests proved the absence of methoxyl groups, methyl pentose sugars, and hexose sugars. The presence of *d*-galacturonic acid, *l*-arabinose, *d*-xylose, and a small amount of an insoluble material was established. From the percentages of

pentosan and uronic acid anhydride given in Table I it is possible to calculate the number of pentose molecules corresponding to each molecule of uronic acid. These values vary from approximately 9 to 36 in the different crops of the mucilage. The corresponding equivalent weights vary from 1300 to 4800. This indicates that *d*-galacturonic acid may be combined with chains of pentose sugars varying in length from 9 to 36 molecules. However, the same result would be obtained if the mucilage were a mixture of a polyuronide and pentosan in varying proportions.

*Hydrolysis of Mucilage*—The mucilage was separated, by hydrolysis with 4 per cent sulfuric acid solution in a bath of boiling water, into three portions; namely, an insoluble X body, the salt of a uronic acid-sugar compound, and a syrup containing the sugars.

*Uronic Acid-Sugar Compounds*—The composition of the uronic acid-sugar compound depends on the duration of hydrolysis of the mucilage. When the heating is for 12 hours, the uronic acid remains combined chiefly with 2 molecules of a pentose sugar. When, however, the heating is for 20 hours, the uronic acid remains combined chiefly with 1 molecule of a pentose.

The presence of a uronic acid was established by the naphthoresorcinol test (3) and the yield of carbon dioxide by the method of Lefèvre and Tollens (4). This was identified as galacturonic acid by the method of Heidelberger and Goebel (5).

The purified calcium salt from hydrolysis of the mucilage for 20 hours gave on analysis 9 per cent CaO and 12.5 per cent CO<sub>2</sub>. A salt composed of 1 molecule of uronic acid and 1 molecule of a pentose sugar should give 8.11 per cent CaO and 12.75 per cent CO<sub>2</sub>. Some of this salt was hydrolyzed in 4 per cent sulfuric acid solution for 14 hours in an autoclave at 120°. From this solution were isolated dextrorotating barium galacturonate (6) and the sugar *l*-arabinose. This established the presence of *d*-galacturonic acid and proved that the sugar attached to it is *l*-arabinose.

The purified calcium salt from the hydrolysis of the mucilage for 12 hours gave on analysis 9.6 per cent CO<sub>2</sub>. The aldehyde group in this salt was oxidized to a carboxyl group by barium hypiodite (7). The calcium salt of the resulting dibasic acid gave on analysis 11.5 per cent CaO, 8.8 per cent CO<sub>2</sub>, and the  $[\alpha]_D^{20} = +69.4^\circ$ . The calcium salt of a uronic acid combined with 2 pentose molecules should give, before oxidation, 9.22 per cent



CO<sub>2</sub> and, after oxidation, 10.93 per cent CaO and 8.6 per cent CO<sub>2</sub>. These data prove that in this salt the *d*-galacturonic acid is combined with 2 pentose molecules. Some of this salt, before oxidation, was hydrolyzed in the autoclave, as already described. In the products of hydrolysis were identified the two sugars, *l*-arabinose and *d*-xylose. These facts prove that *d*-xylose is the second sugar in the chain attached to the *d*-galacturonic acid.

*The Sugars*—The gummy sugar obtained by hydrolysis of the mucilage in a bath of boiling water generally amounted to between 75 and 90 per cent of the weight of the mucilage used, depending on the duration of heating and the crop of mucilage used. In general, hydrolysis for 12 hours gave slightly lower weight of gum sugar than hydrolysis for 20 hours.

When the gum sugar obtained by hydrolysis for 12 hours was completely freed of salts by use of absolute alcohol and was then dissolved in glacial acetic acid, it was converted very largely into crystalline *d*-xylose which melted at 149° and had  $[\alpha]_D^{20} = +18.5^\circ$ . This sugar was further identified as *d*-xylose by Bertrand's test (8), which gave the characteristic boat-shaped crystals of  $\text{Cd}(\text{C}_6\text{H}_5\text{O}_6)_2 \cdot \text{CdBr}_2 \cdot 2\text{H}_2\text{O}$ . This gummy sugar seemed to be composed almost wholly of *d*-xylose.

When the gum sugar obtained by hydrolysis of the mucilage for 20 hours was treated as described above, approximately 80 per cent of it was obtained as crystalline *d*-xylose. The remaining gum gave qualitative tests for both *d*-xylose and *l*-arabinose. In one case crystalline *l*-arabinose, melting at 154° and showing  $[\alpha]_D^{20} = +104^\circ$  was obtained in small amounts from the gum sugar after removal of most of the *d*-xylose. This was further identified as *l*-arabinose by conversion to the diphenylhydrazone melting at 199°. It thus appears that longer heating leads to splitting off of some of the *l*-arabinose from the *d*-galacturonic acid.

The pentosan portion of this mucilage consists very largely of *d*-xylose, with a small amount of *l*-arabinose.

*X Body*—The insoluble material remaining after hydrolysis of the mucilage amounted to between 1.5 and 2.5 per cent of the mucilage used, depending on the method of preparation of the mucilage. It will be referred to as an *X* body.

In general, when a polyuronide is hydrolyzed, it leaves varying

amounts of insoluble material. This sometimes amounts to 20 per cent of the weight of the polyuronide. Occasionally the decided change in the appearance of the solution as hydrolysis proceeds suggests that the insoluble material is a definite part of the polyuronide molecule (9). Possibly in such cases this material corresponds to the aglucone part of such glucosides as quercetrin. In other cases the small amount of insoluble material may be due to impurities or to decomposition products formed during hydrolysis.

*Ash*—The mucilage is present as a salt of one or more metals. The ash resulting from ignition of the mucilage gave qualitative tests for potassium, calcium, iron, and phosphate.

*Seed Coats*—The seed coats contain all the mucilage and very little else. When they are removed from the seed and mixed with water, they act as the mucilage does. On hydrolysis they yield approximately the same amounts of the substances as the mucilage. Analysis of the seed coats gave the following results, expressed in per cent: ash 1.99, total nitrogen 0.18, ether extract 0.38, crude fiber 2.25, pentosan 86.5, carbon dioxide 2.15, uronic acid anhydride 8.6. In this analysis the X body would appear as a part of the crude fiber. The per cent mucilage in the seed coats would be the sum of the percentages of the ash, uronic acid anhydride, pentosan, and a part of the crude fiber. This amounts to approximately 98 per cent of the weight of the seed coats.

*Structure of Mucilage*—Little is known of the exact structure of this mucilage. However, the *d*-galacturonic acid is combined, probably through its aldehyde group, with a molecule of *l*-arabinose. The latter sugar is combined, through its aldehyde group, with a chain of varying numbers of molecules of *d*-xylose. Apparently the X body is combined with a molecule of *d*-xylose.

#### SUMMARY

The mucilage from psyllium seed consists of a mixture of polyuronides. These bodies are composed of *d*-galacturonic acid combined with *l*-arabinose. The latter sugar is combined with chains of *d*-xylose molecules varying in length from 8 to 35 molecules and a small amount of an X body.

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## OXYGEN DISSOCIATION CURVES OF BIRD BLOOD

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In a recent review Redfield (1) has called attention to the atypical manner in which the blood of certain birds and fish combines with oxygen. This matter aroused our interest because in such remotely related animals as *Urechis* (2), the skate (3), four reptiles (4-6), and the dog (7) the form of the oxygen dissociation curve is roughly similar to that of human blood. Accordingly, experiments have been carried out on six species of birds, including those studied by Wastl and Leiner (8). In order to make a complete study on a single specimen of blood, enough birds were decapitated to yield at least 100 cc. of blood. Such a specimen with heparin added remains virtually unchanged during 48 hours storage at 0°; actually our experiments were usually completed within 24 hours.

There is a slight but appreciable error if the usual method of equilibration is applied to blood having nucleated red cells. Oxygen is being utilized continuously; hence, a moving rather than a stationary equilibrium is reached. We sought to avoid this error by the use of hemolyzed red cells.<sup>1</sup> With human blood one thus obtains a solution which is fairly homogeneous, yields very little sediment on centrifugation, and gives an oxygen dissociation curve almost identical in form and position with that of whole blood if proper pH adjustment is made. Bird blood thus treated yields a heterogeneous mass which separates into two phases on

<sup>1</sup> Blood was centrifuged, supernatant plasma and white cells were removed, and the red cells washed three times with 1 per cent NaCl solution. The addition of 2 volumes of distilled water at room temperature soon completes hemolysis. The further addition of 1.2 mg. of NaHCO<sub>3</sub> to each ml. of solution restores the available base to a value about equal to that in the original blood.

centrifugation. The upper half or two-thirds is a homogeneous solution of hemoglobin; the lower portion is a reddish jelly. On washing the jelly with distilled water, its volume increases and some hemoglobin appears in the wash water.

Oxygen dissociation curves have been derived for the supernatant homogeneous solution as well as for the intact whole blood. It was found, however, that such a solution has much less affinity for oxygen than blood, even when pH is taken into account.

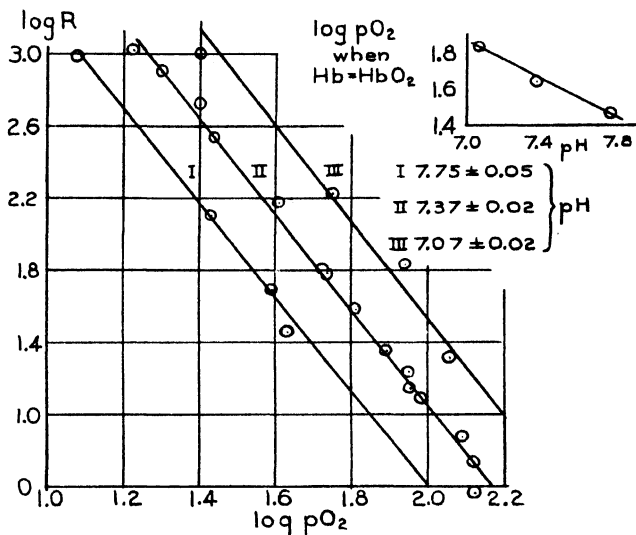


FIG. 1. Hemoglobin solution from blood of the domestic fowl at 37.5°. Left-hand figure,  $\log pO_2$  as a function of  $\log R$  where  $R = 100 Hb/HbO_2$ . Right-hand figure, the relation between the position of the oxygen dissociation curve and pH. The unit of measure for  $pO_2$  is mm. of Hg.

The evidence for this is found in Figs. 1 and 2. The dissociation curves for hemoglobin solution at three pH values are given in Fig. 1, together with the relation between pH and position of the curves at one-half saturation. Similar experiments were carried out on whole blood, and in Fig. 2 a comparison is made between hemoglobin solution and whole blood, the pH of the solution as well as of the red cells being 7.1.

Various hypotheses may be advanced to explain this curious

behavior. A plausible one is that two hemoglobins are present in intact red cells of birds and that a partial separation is effected by our procedure. The existence of two hemoglobins in mammalian blood has been suggested by Brinkman, Wildschut, and Wittermans (9) and in the blood of the young domestic fowl by Hall (10). Another possibility is that partial denaturation or coagulation has occurred. However the phenomenon is explained, the fact remains that from the physiological point of view the nature of the oxygen dissociation curve can best be studied in whole blood.

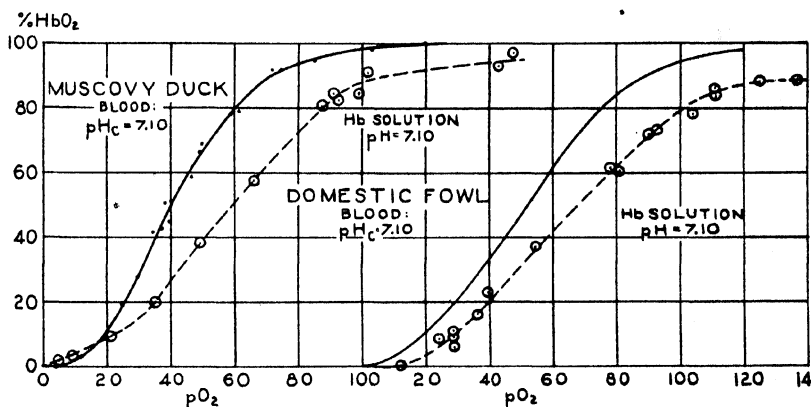


FIG. 2. Oxygen dissociation curves of blood and hemoglobin solutions at 37.5°. (The data for the blood of the domestic fowl will be published at another time.)

The presentation of data on blood has been simplified by converting all points to a  $pH_c$  value of 7.1. In order to accomplish this, the blood was equilibrated at  $pCO_2$  values of 10, 40, and 100 mm. and total  $CO_2$  as well as total  $O_2$  determined for each point. The distribution of bicarbonate between cells and serum was found to be about the same as in human blood. The ionic strength also is about the same; hence it has been assumed that the  $pK'$  values calculated for mammalian red cells by Stadie and Hawes (11) are applicable.

It is natural to wonder whether this assumption could be sufficiently in error to account for the difference between the curves

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for blood and hemoglobin. This seems improbable, for in our hemoglobin solutions the  $pK'$  was experimentally determined, with

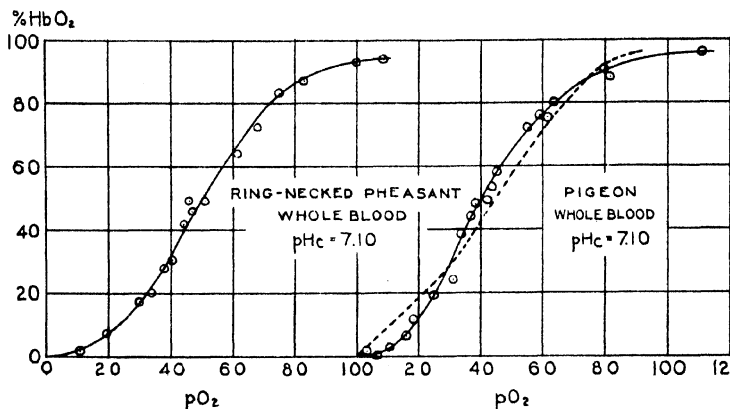


FIG. 3. Oxygen dissociation curves of blood at 37.5°. The broken line corresponds to the curve of Wastl and Leiner (8) for the pigeon blood at 42° and  $pCO_2$  40 mm. of Hg.

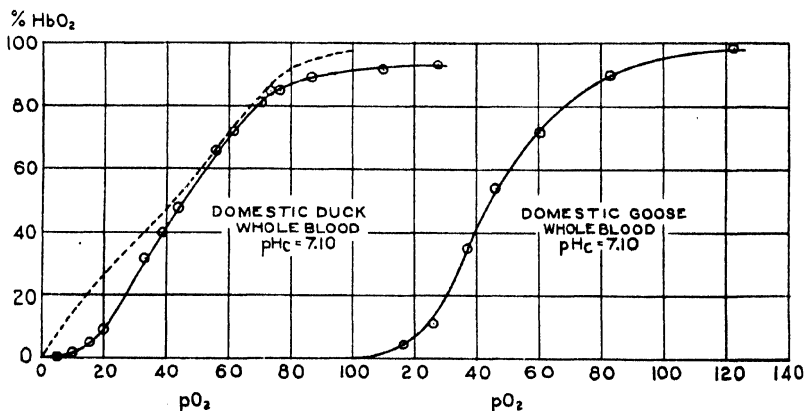


FIG. 4. Oxygen dissociation curves of blood at 37.5°. The broken line corresponds to the curve of Wastl and Leiner (8) for duck blood at 42° and  $pCO_2$  40 mm. of Hg.

the glass electrode, and found to be within a few hundredths of the expected value; a difference of 0.3 would be required to bring the oxygen curves together.

The oxygen dissociation curves for four other species of birds are shown in Figs. 3 and 4. These all differ from the curve characteristic of human blood in two respects: there is much less affinity for oxygen, as Wastl and Leiner report (8), and the first increments in oxygen pressure appear to be associated with virtually no uptake of oxygen. This last observation is presumably a consequence of the methodical error referred to above, for in this part of the curve its effect is most evident.

Aside from the lower portion of the curves there is nothing unusual about their form; they all may be considered typical oxygen dissociation curves. The peculiarities found by Wastl and Leiner (8) for duck and pigeon blood are not supported by our study of the same species. We are of the opinion that their results are complicated by the fact that observations were made on two or more specimens of blood. Although  $p\text{CO}_2$  was held constant, there was no control of the alkaline reserve in the different specimens and no assurance, therefore, of constant pH.

#### SUMMARY

The product of hemolysis of bird erythrocytes with water is not homogeneous. The supernatant solution of hemoglobin obtained from this mixture by centrifugation has much less affinity for oxygen than whole blood, even if proper pH adjustment is made.

Bird blood has a lower affinity for oxygen than the blood of man, but the form of the oxygen dissociation curve is much the same.

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# THE NATURE OF THE DIFFERENCE IN PHOSPHOLIPID CONTENT OF OXALATED AND HEPARINIZED PLASMA

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(Received for publication, February 6, 1935)

Man and Gildea (1) observed that the total fatty acid content of either serum or heparinized plasma was greater than that of oxalated plasma. This difference was tentatively ascribed to one of two causes: either a precipitation of plasma phospholipids by the sodium or potassium oxalate (MacLean and MacLean (2)), or an increase in the plasma volume of oxalated blood due to shrinkage of the red blood cells.

Since our plasma phospholipid studies involved the use of oxalated blood, it was of interest to determine in what manner and to what extent addition of potassium oxalate modified plasma phospholipid content. The present experiments have demonstrated that the phospholipid content of oxalated plasma is consistently lower than that of heparinized plasma; this difference is due to alterations in cell and plasma volumes and not to phospholipid precipitation.

## EXPERIMENTAL

Solutions of heparin (Hynson, Westcott and Dunning) and potassium oxalate (neutralized according to Peters and Van Slyke (3)) were added to venous blood drawn from normal humans, dogs, and rabbits. The final concentration of heparin in the blood was always 0.04 per cent; that of potassium oxalate was varied between 0.1 and 0.8 per cent.

Whole blood and plasma lipids were extracted by a modification of the Bloor (4) procedure; 2 cc. were extracted with two 50 cc. portions of a boiling mixture of 3 parts of absolute ethyl alcohol and 1 part of absolute ethyl ether. The phosphorus in this

extract, soluble in petroleum ether, was determined by the Fiske and Subbarow (5) method. The experimental error was  $\pm 1.5$  per cent.

Plasma and cell volumes were determined by centrifuging the freshly drawn blood in a 12 cc. calibrated centrifuge tube at 2500

TABLE I

*Comparison of Phospholipid Phosphorus Content of Blood, without Any Anticoagulant, Heparinized, and Oxalated*

The results are expressed in mg. per 100 cc. of blood.

Subject No.	Blood without anti-coagulant	Heparinized blood	Oxalated blood				
			Concentration of K oxalate, per cent				
			0.1	0.2	0.4	0.6	0.8
Human 1	9.45	9.36	9.4	9.4		9.4	9.4
" 2	10.8	10.8		10.7	10.8	10.7	10.8
" 3	10.9	10.8		10.8	10.9	10.7	10.9
" 4		12.1		12.0	12.1	11.8	12.1
" 5	11.3		11.3	11.5	11.4		11.3
" 6		8.19		8.1			
" 7		9.17		9.2			
" 8	11.1			11.0			
Dog 1		13.9		13.7			
" 2		14.0		14.1			
" 3		13.6		13.7			
" 4		14.0		13.7			
" 5		13.2		13.1			
" 6		13.9		14.2			
Rabbit 1		9.68		9.75			
" 2		8.62		8.60			
" 3		9.12		8.92			
" 4		8.55		8.70			

R.P.M. until constant cell volumes were obtained. This required not longer than 70 minutes.

*Whole Blood*.—The phospholipid phosphorus content of whole blood was determined in eight humans, six dogs, and four rabbits. Samples treated in three ways were analyzed: (1) without any anticoagulant, (2) heparinized, and (3) oxalated (0.1 to 0.8 per cent potassium oxalate). As Table I shows, none of these treatments led to a significant difference in phospholipid content.

TABLE II

*Comparison of Plasma Volume and Phospholipid Phosphorus Content of Plasma from Oxalated and Heparinized Blood*  
 The values for plasma volume are expressed in per cent; those for phospholipid phosphorus in mg. per 100 cc. of plasma.

Subject No.	Plasma volume				Phospholipid phosphorus									
	Heparinized	Oxalated				Heparinized	Found				Corrected*			
		Concentration of K oxalate, per cent					Oxalated				Oxalated			
							K oxalate, per cent				K oxalate, per cent			
		0.1	0.2	0.3	0.4		0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4
Human 1	48.8	52.4			6.95		6.50					7.00		
" 2	50.6	54.6			5.45		5.15					5.55		
" 3	48.7	54.2			8.52		7.70					8.60		
" 4	51.9	55.4	55.7	55.4	8.00		7.40	7.40	7.40	7.85	7.95	7.90	7.92	
" 5	54.2	56.0	59.7	59.3	8.85		8.05	8.10	8.10	8.80	8.85	8.85	8.90	
" 6	50.5	52.8	55.9	55.3	6.06		5.63	5.30	5.33	5.90	5.87	5.84	5.89	
" 7	52.0	56.4	57.7	57.4	10.2	9.20	9.05	9.10	9.10	10.0	10.0	10.1	10.1	
Dog 1	43.5	48.5			11.2		10.3				11.5			
" 2	46.1	51.0			13.5		12.4				13.7			
" 3	45.5	49.5			14.0		12.6				13.7			
Rabbit 1	54.9	59.1			5.48		5.03				5.42			
" 2	57.6	62.3			4.11		3.86				4.18			

\* Corrected phospholipid phosphorus =  $\frac{\text{oxalated plasma volume}}{\text{heparinized plasma volume}} \times \text{mg. of phospholipid phosphorus in oxalated plasma.}$  cc. of

*Plasma*—However, Table II shows that there was a difference in plasma. The phospholipid phosphorus content of oxalated plasma was in every instance lower than that of heparinized, the difference varying from 4 to 12.5 per cent. Table II also shows that the plasma volume of oxalated blood was greater than that of heparinized, confirming the earlier observation of Eisenman (6). When the phospholipid phosphorus content of oxalated plasma is corrected for this difference in plasma volume, the resulting values approximate those obtained with heparinized plasma.

Table II demonstrates two other points of interest. First, increasing the concentration of potassium oxalate from 0.2 to 0.4

TABLE III

*Effect of Addition of Potassium Oxalate on Phospholipid Phosphorus Content of Heparinized Plasma*

The results are expressed in mg. per 100 cc. of plasma.

Subject No. (human)	Heparinized plasma	Heparinized plasma + K oxalate					
		Concentration of K oxalate, per cent					
		1	2	4	6	8	10
1	6.94	7.02	7.02	7.05	6.05		
2	8.12	8.07	8.08	8.04	8.08	8.07	7.75
3	6.64	6.8	6.47	6.66	6.57	6.51	6.54
4	6.37	6.32			6.33		6.26
5	7.34				7.23		7.24
6	8.50				8.57		8.65

per cent had little effect on either phospholipid content or plasma volume. Second, the phospholipid content of heparinized plasma was uniformly from 10 to 12 per cent higher than that of plasma containing 0.2 to 0.4 per cent potassium oxalate.

These results indicate that the difference in phospholipid content of oxalated and heparinized plasma is due to a change in plasma volume and not to phospholipid precipitation.

Additional evidence that such is the case was given by experiments in which varying amounts of potassium oxalate were added to heparinized human plasma, the final potassium oxalate concentration varying from 1 to 10 per cent. The results, summarized in Table III, show that even such high concentrations do not precipitate plasma phospholipids.

## SUMMARY

The phospholipid content of heparinized plasma is consistently 10 to 12 per cent higher than that of oxalated plasma, when the latter contains from 0.2 to 0.4 per cent potassium oxalate. This difference is the result of alterations in cell and plasma volumes produced by the oxalate; the volume of plasma from heparinized blood is correspondingly less than that obtained from oxalated blood.

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## LACTIC ACID FORMATION IN LIVER\*

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(Received for publication, February 15, 1935)

The concentration of lactic acid in and the formation of lactic acid by tissues *in vitro* have been studied by many investigators. While it is known (Rosenthal (1), Warburg, Posener, and Negelein (2)) that liver in common with many other tissues forms lactic acid, it has not been sufficiently emphasized that, unlike muscle, the liver is easily damaged so that lactic acid formation may not occur.

*Determination of Lactic Acid in Liver*—The determination of lactic acid in liver tissue by a method involving the formation and estimation of acetaldehyde is complicated on account of the presence of relatively high concentrations of carbohydrate material. The complication is especially great when liver tissue is analyzed after digestion *in vitro*. On account of difficulties which were encountered in the use of the method of Friedemann, Cotonio, and Shaffer (3), we have studied its application to the analysis of liver tissue in some detail. In comparison with the Friedemann, Cotonio, and Shaffer method (with  $\text{KMnO}_4$  and  $\text{H}_2\text{SO}_4$ ) the modification of Friedemann and Kendall (4) (with  $\text{MnO}_2$  and  $\text{H}_3\text{PO}_4$ ) was studied. Both yielded results  $98 \pm 2$  per cent of the theory when standard solutions of pure lactic acid or zinc lactate were used. 0.001 N iodine was employed in the titration and 0.5 to 1.5 mg. of lactic acid were taken for each determination.

From 5 to 10 gm. of hashed or sliced liver were suspended in an

\* The data in this paper were taken from the thesis presented by Phyllis A. Bott to the Faculty of the Graduate School of the University of Pennsylvania, in 1930, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



approximately equal weight of salt solution.<sup>1</sup> After standing for 15 minutes or longer, mercuric chloride and hydrochloric acid were added and the solution was allowed to remain in the refrigerator overnight (Schenk procedure). The final volume of the solution was 10 or more times the weight of the tissue used.

The livers of well fed animals were so rich in glycogen that the protein-free filtrate, especially when the livers had been ground, was turbid with glycogen which prevented the flocculation of mercuric sulfide on the addition of hydrogen sulfide. Flocculation was brought about by boiling the solution for a minute or two with the addition of a pinch of sodium sulfate after saturating with hydrogen sulfide.

Carbohydrate material was removed by treating the neutralized filtrate with copper sulfate and calcium hydroxide. However, such amounts of copper sulfate and calcium hydroxide as are generally used for blood or muscle filtrates were inadequate for the removal of reducing substances from the liver filtrates of the type described. This was especially true if the solutions had been heated. It was found necessary to use as much as 35 cc. of 10 per cent copper sulfate solution and 40 cc. of a 5 per cent suspension of calcium hydroxide for every 20 cc. portion of neutralized protein-free filtrate (corresponding to about 1.6 gm. of liver). These amounts were determined by increasing the volume of reagents used in repeated trials, until the resulting filtrates no longer reduced Benedict's reagent. Since it was impossible to predict the amount of the reagents required for each precipitation, these large quantities of reagents were used routinely where the copper-lime method was employed with liver.

When lactic acid was determined on copper filtrates from liver prepared as described above, it was noticed that in many cases, as the end-point of the titration was approached, the color appeared and then faded rapidly and a considerable additional titration was necessary to produce a color which persisted for 30 seconds. The effect was that which would be produced by the presence of material which dissociated from bisulfite slightly less readily than

<sup>1</sup> 0.9 per cent NaCl, 2 per cent bicarbonate, and bicarbonate-Ringer's solution were used in various digestion experiments. The initial samples were suspended in the same fluid as the samples which were allowed to digest.

does acetaldehyde. The fading was more pronounced when permanganate and sulfuric acid were used for oxidation. These observations indicated that some interfering substance was present in liver, not entirely removed by the copper-lime treatment. It was therefore decided to study a method involving ether extraction.

In order to study the recovery of lactic acid, 40 cc. of lactic acid solution were placed in an efficient continuous extractor, the solution saturated with ammonium sulfate, 6 drops of concentrated sulfuric acid were added, and the solution was extracted with 100 cc. of ether. Extraction was continued for various periods of time at such a speed that ether dropped from the condensers in an almost continuous stream. When extraction was discontinued, evaporation was carried out as described by Clausen (5) except that it was not found necessary to evaporate "almost to dryness." After evaporation of the ether, the neutralized aqueous solutions, which were then transferred to 15 cc. beakers, were heated on a water bath for about 45 minutes during which time about half of the water evaporated, leaving approximately 5 cc. of solution. This was transferred to a 50 cc. volumetric flask and made up to volume. As a rule 10 cc. aliquots served for lactic acid determinations.

Anhydrous ether containing only 0.5 per cent of alcohol was used for extraction. Blanks were run on this ether, evaporation on a water bath being performed as described above. The 10 cc. aliquots produced blanks no larger than those obtained with the reagents used in the lactic acid determination itself (0.3 cc. of 0.001 N iodine). If ordinary U.S.P. ether was used, a high blank (1.1 to 1.4 cc. of 0.001 N iodine) was obtained, but this could be reduced to about 0.4 cc. by shaking the ether immediately before using, with dilute alkaline potassium permanganate solution, and then twice with small portions of water.

By the method described above we have been able to obtain quantitative recoveries of 1.26 to 44.2 mg. of lactic acid in 2.5 hours of extraction. A summary of data is as follows: Extractions for 2.5 to 8.0 hours yielded recoveries of 100 per cent; extractions for 2 hours yielded 94 to 98 per cent; extractions for 1.5 hours yielded 81 to 93 per cent. The percentage recoveries were calculated from determinations made directly on the solutions which yielded  $98 \pm 2$  per cent of the theoretical values.

Solutions from tissues were always extracted for at least 3 hours. There is apparently some danger in extracting for very long periods of time (as for example, 30 hours as recommended by Embden and Kraus (6) for more concentrated extracts), because on keeping the acid aqueous solution in contact with ether for many hours, the apparent lactic acid content was sometimes raised. This was true even when the solutions were kept overnight in the refrigerator. Our observations are not in accord with those of Friedemann and Graeser (7) who dismiss ether extraction with the statement that

TABLE I

*Recovery of Added Lactic Acid from Suspensions of Liver Hash*

The results are expressed as mg. per 100 gm. of fresh liver.

Experiment No.	Lactic acid found in liver	Lactic acid added	Total lactic acid found	Added lactic acid recovered	Per cent recovery	Method
38	93	14	98	5	35	Cu-lime*
	95	14	98	3	21	" †
	75	14	88	13	93	Ether*
	75	14	88	13	93	" †
40	23	43	66	43	100	Cu-lime*
	25	43	71	46	107	" †
	28	43	69	41	95	Ether*
	28	43	70	42	97	" †
53	106	50	154	48	96	Cu-lime*
	107	50	156	49	98	" †
	93	50	144	51	102	Ether*
	93	50	143	50	100	" †

\* With  $\text{MnO}_2 + \text{H}_3\text{PO}_4$ .

† With  $\text{KMnO}_4 + \text{H}_2\text{SO}_4$ .

there is always loss of lactic acid due to oxidation by organic peroxides formed during extraction and evaporation and due to volatilization during evaporation of aldehydes which are always present.

Table I shows the recovery of added lactic acid from suspensions of hashed liver as determined on copper-lime filtrates and on ether extracts. When ether was used the proteins were precipitated by the Schenk procedure and the filtrate extracted after saturation with ammonium sulfate and addition of sulfuric acid. In calculating the results, total volumes were calculated on the basis of

the volumes of material used, the specific gravity of liver being taken as 1. The recoveries of added lactic acid are shown to vary from 93 to 102 per cent after ether extraction. The recoveries by the copper-lime procedure varied from 21 to 107 per cent. The differences between the analyses by the two procedures are perhaps best shown by the data in the second column. Here, as with many other comparisons which are not reported, the results of the copper-lime procedure ranged from 14 per cent lower to 25 per cent higher than those of the ether extractions. Our experience with many analyses leads us to conclude that the copper-lime procedure is unsatisfactory and that good results can be obtained by ether extraction.

It was not found necessary to use ether extraction on filtrates from muscle. 4 cc. of copper sulfate solution and 4 cc. of calcium hydroxide suspension were ample to remove the sugar from 20 cc. of muscle filtrate. Good end-points were obtained when lactic acid was determined on the copper filtrates, and the results of these determinations agreed very closely with those on ether extracts.

*Comparison of Lactic Acid Formation in Hashed and Sliced Liver and Muscle*—In the absence of oxygen, considerable lactic acid formation occurs in intact striated muscle as well as in muscle hash or press juice. Hashing, freezing, and pulverizing do not appear to interfere much with the formation of lactic acid by striated muscle. The ease with which lactic acid may be formed in suspensions of striated muscle tissue prepared in different ways has led various investigators to use similar procedures for the study of lactic acid formation in other tissues. The results have been to a considerable extent misleading. Experiments recorded below demonstrate the great importance of intact cell structure for lactic acid formation in liver.

Data in Table II demonstrate that hashed liver tissue forms lactic acid much more slowly than do liver slices. In contrast to this, hashed and sliced muscle tissue form lactic acid at about the same rate.

There is not the rapid formation of lactic acid in liver that there is in muscle after it is removed from the body. A portion of liver dropped into liquid air as soon as it was removed from the animal was compared with a portion taken to the cold room and ground

in a meat chopper as soon as possible. Both portions showed the same concentrations of lactic acid.

In other experiments not described in detail we have found the lactic acid concentrations in hashed livers of well fed rabbits to range from 70 to 150 mg. per cent before incubation,<sup>2</sup> and after incubation to range from 134 to 300 mg. per cent. These results are similar to those of Riesser and Hansen (8) and Krause (9). The livers of fasting animals in our experiments contained 20 to 50 mg. per cent of lactic acid which about doubled after 4 hours

TABLE II

*Comparison of Lactic Acid Formation in Hashed and Sliced Tissue of Rabbits*  
Digestions in bicarbonate-Ringer's solution at 37°.

	Experiment No.	Temperature during preparation	Lactic acid per 100 gm. fresh tissue			Ratio of increase in slices to increase in hash
			Initial	Final		
				Hashed	Sliced	
		°C.	mg.	mg.	mg.	
Liver, 2 hrs. in N <sub>2</sub>	52	25	117	168*	225	2.08
“ 30 min. in O <sub>2</sub> +	44	3	114	161*	481	8.00
2 hrs. in N <sub>2</sub>	46	3		181	285	
Same	49	3		164	259	
“	50	25	111	222	372	2.35
“	51	25	128	238	406	2.53
Muscle, 2 hrs. in vacuum	46	3	297	846*	821	0.95
Muscle, 2 hrs. in N <sub>2</sub>	49	3	581	947*	1012	1.18

\* Put through meat chopper several times.

digestion. It is interesting to note that the livers of well fed animals contained more lactic acid initially and formed more lactic acid during digestion than did livers of fasting animals.

*Effect of Freezing Liver on Its Glycolytic Activity*—The effects of freezing and pulverizing and of freezing alone previous to the incubation of liver are shown in Table III. In Experiments 20 to 38 liver was powdered in a mortar after being frozen for about 10 minutes with liquid air or carbon dioxide snow. In Experiments 50 and 51 liver was sliced and then frozen for about 10 minutes.

<sup>2</sup> These high initial values are explained in the following paper.

In all cases, little or no lactic acid was formed on incubation. That the unfrozen tissue could form lactic acid is shown in the last two columns of Table III.

It is concluded that treatment which damages the structure of liver tissue decreases its capacity for lactic acid production. This may explain the results of numerous experiments involving liver autolysis (Sevringhaus, Koehler, and Bradley (10), Rona and associates (11, 12)) in which the liver as a rule was ground to a pulp and treated with an antiseptic and was sometimes previously frozen. In these experiments there was usually only a small amount of lactic acid produced over a period of several days.

TABLE III

*Effect of Freezing on Lactic Acid Formation in Liver Tissue of Rabbits*

The results are expressed as mg. of lactic acid per 100 gm. of fresh liver.

Experiment No.	Temperature during preparation °C.	Initial	Freezing medium	Frozen		Incubation	Not frozen	
				Ground	Sliced		Ground	Sliced
20	3	104	CO <sub>2</sub> snow	100		Vacuum, 2 hrs. at 25°		
29	3	75	Liquid air	76		Vacuum, 2 hrs. at 25° + 2 " "		
						37°		
35	3	99	" "	88		" "	301	
38	3	75	" "	78		" "	185	
50	25	111	" "		120	30 min. in O <sub>2</sub> , 2 hrs. in N <sub>2</sub> , all at 37°	222	372
51	25	128	" "		127	" "	238	406

The loss of power, by liver, to form lactic acid after being frozen by liquid air and pulverized was overlooked by Simpson and Macleod (13). In order to compare carbohydrate metabolism of liver and muscle each tissue was frozen with liquid air, pulverized, and then allowed to stand at room temperature in salt solution. They found that, whereas glycogen disappeared from both tissue suspensions, lactic acid was formed by the muscle alone. The liver suspension formed reducing sugar but not lactic acid. The conclusion was drawn that muscle functions by forming lactic acid and no sugar, whereas liver forms sugar and no lactic acid. The present investigation demonstrates that the lack of formation of

lactic acid by liver in the experiments of Simpson and Macleod was probably due to the fact that the lactic acid-forming system had been destroyed by freezing and grinding. While an important function of the liver *in vivo* may be to form sugar from its glycogen stores, it is nevertheless true that liver is capable of forming considerable amounts of lactic acid.

That other tissues are similar to liver and unlike striated muscle with respect to the relation of cell structure to lactic acid production is suggested by the studies of Warburg, Posener, and Negelein (2), Barr, Ronzoni, and Glaser (14), and Ronzoni (15). Indeed, it seems that the lactic acid production of striated muscle is rather peculiar in its relative independence of tissue structure.

#### SUMMARY

A comparison of methods for the preparation of liver extract for lactic acid analysis has been presented. Errors were shown to occur when the copper-line procedure was used. Ether extraction was found to be more satisfactory.

Ground liver tissue produced less lactic acid than slices of liver. Freezing with liquid air destroyed the glycolytic activity of liver.

Muscle hash and slices produced lactic acid with about equal rapidity.

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## THE CONCENTRATIONS OF LACTIC ACID IN BLOOD AND LIVER OF RABBITS

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In experiments described in the previous paper we observed that the concentrations of lactic acid in livers of well fed animals (generally 70 to 150 mg. per cent) were higher than those of fasting animals (20 to 50 mg. per cent). Cori (1) noted a similar variation in mice. As some struggling occurred when the rabbits were tied down and anesthetized, it was assumed that the high concentration of lactic acid in the liver might be associated with an increased lactic acid concentration of blood resulting from heightened lactic acid production. It is known that the liver removes lactic acid from blood after exercise. We therefore thought it worth while to study the relation between the concentrations of lactic acid in blood and liver of well fed and fasting rabbits after exercise.

The animals were tied on an animal board, sodium amytal was given intraperitoneally, blood samples were taken from an ear vein, and, when anesthesia was complete, liver samples were obtained. Rabbits are so excitable that the amount of muscle activity which took place was probably far greater than would have been encountered with other common laboratory animals.

About 2.5 cc. of blood were drawn, usually by nicking the ear vein, into a paraffined test-tube immersed in ice water. 2 cc. were quickly pipetted into a flask containing 10 cc. of water. The usual Schenk and Van Slyke precipitations were made and lactic acid determined in the final filtrate.

The samples of liver were dropped into liquid air immediately after removal from the animal and powdered in an iron mortar. From 2 to 3 gm. were weighed (in the cold room) into flasks containing 15 cc. of Ringer's solution and 3 cc. of 8 per cent HCl.



After standing 1 hour in the cold room 12 cc. of 8 per cent HCl and 30 cc. of 5 per cent HgCl<sub>2</sub> were added. The solutions were filtered

TABLE I  
*Blood and Liver Lactic Acid of Well Fed and Fasting Rabbits*

Experi- ment No.	Condition of animal	Position during anesthesia	Time after injection of amytal	Blood lactic acid	Liver lactic acid
			<i>min.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 gm.</i>
1	Fed	Tied on board	45	81	60
2	"	" " "	16	138	
			18		95
3	"	" " "	0	164	
			25	115	
			54	76	
			56		37
			90	53	
			91		27
4	"	" " "	8	95	
			25	68	
			27		36
5	"	" " "	7	88	
			28	51	
			33		45
6	"	" " "	6	157	
			18	150	90
7	"	" " "	7	186	
			17	176	74
8	"	Upright in cage	30	43	30
9	"	" " "	83	44	
			85		40
10	Fasted 6 days	Tied on board	7	106	
			31	76	
			35		44
11	" 5 "	" " "	15	36	
			45	32	24
12	" 8 "	" " "	10	50	
			22	47	35

the next morning and lactic acid determined by ether extraction on the filtrates.

The data for the concentrations of lactic acid in blood and liver

are given in Table I. It is evident that the concentration of lactic acid was raised to a high level in the blood of most of the well fed animals. The blood samples taken early in the experiments from three fasting animals showed smaller accumulations of lactic acid than the average of the well fed animals. Two of the three fasting animals had especially low concentrations of blood lactic acid.

The most obvious explanation for the difference in levels of blood lactic acid between the two groups of animals is that the fasting animals were less active than the well fed animals and therefore formed less lactic acid. However, we could observe little or no difference in the amount of muscle activity between the two groups of animals.

The animals of Experiments 8 and 9, which were well fed, were given sodium amytal intraperitoneally, with as little disturbance as possible, and were allowed to sit in the cage until anesthetized. Then samples of blood and liver were taken for analysis. As both animals had low concentrations of lactic acid in blood and liver, it is concluded that muscle activity due to excitement was the cause for the high concentrations of lactic acid in the blood of the other well fed animals.

Samples of liver were taken from all animals as soon as anesthesia was sufficiently deep. It is significant that in all instances the concentration of lactic acid in liver was less than it was in blood. In a number of experiments the differences were considerable, especially when the blood lactic acid was high. Experiment 3 shows that the differences were not caused by a lag in the accumulation of lactic acid in liver due to slow diffusion from the blood. In this experiment the concentration of lactic acid in liver decreased as the concentration in the blood decreased and the concentration in liver continued to be far below that in blood. The differences are much greater than could be explained on the basis of the different water contents of tissue and blood plasma.

Glycogen increases in liver after administration of lactic acid (2). By studying the blood going to and from the liver it has been demonstrated that the liver takes up lactic acid from blood after exercise (3, 4). Presumably it is converted into glycogen. Our own observations show that lactic acid does not accumulate to any great extent in the liver when it disappears from the blood after exercise, the lactic acid concentrations in the liver remaining

considerably below the blood levels. Therefore it would appear that the synthetic processes in the liver are capable of forming glycogen from lactic acid with considerable rapidity.

#### SUMMARY

The concentration of lactic acid in the blood of well fed rabbits may rise to very high values following moderate struggling and anesthetizing with amytal. The rise is not so great in fasting animals.

The concentration of lactic acid in liver rises and falls with changes in the blood but is usually far below the blood level.

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# THE MEASUREMENT OF pH AND ACID-NEUTRALIZING POWER OF SALIVA

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The reaction of human saliva has been determined by a large number of investigators. Indeed, the earlier experiments in this direction antedate the quantitative formulation of acidity in terms of pH. The hydrogen electrode was found to give results which differed by 0.1 to 0.5 pH unit from those obtained with the aid of indicators (1). There appears to be no other published evidence regarding the precision of any of the methods used. Measurements of greater precision than the indicators afford would be desirable, if the behavior of such saliva constituents as proteins, phosphates, etc., is to be studied. The above considerations led us to investigate the applicability to saliva of those electrometric methods which have been successfully employed in the case of blood serum and plasma.

The subjects were 20 to 45 years of age. Active caries was present to a slight extent in a few cases. Otherwise the oral conditions were normal. The mouth was rinsed with water before collecting the sample. The saliva was allowed to accumulate in the mouth (by normal flow unless otherwise specified) for a sufficiently long period, then transferred to a suitable vessel.

In reporting the results of our measurements, we shall use the term pH in its empirical sense only (2); *i.e.*, without any implication concerning its relationship to H ion concentration or activity.

## EXPERIMENTAL

*Hydrogen Electrode Checked against Quinhydrone Electrode*—The measurements were performed at  $25^{\circ} \pm 0.05^{\circ}$ . In using the capillary quinhydrone electrode, we followed the procedure described by Laug (3).

The saliva was collected for 25 to 30 minutes and transferred to the solution cup of the Clark electrode vessel. The cup was closed with a rubber stopper which carried two glass tubes. One communicated, through a stop-cock, with a pressure reservoir. The other, a capillary dipping into the saliva, was also provided with a stop-cock.

The saliva was mixed, in so far as possible, by rocking the vessel. A quinhydrone cell was then attached to the capillary and filled. The measurement with the quinhydrone electrode was repeated at intervals until the difference between two consecutive results did not exceed 2 millivolts; then a check determination was made with the hydrogen electrode.

In calculating the pH values, the quinhydrone electrode was assumed to be more positive than the hydrogen electrode by 0.6992 volt at 25°. The quinhydrone electrode showed a drift of about 1 millivolt during 3 to 8 minutes after the filling; equilibrium readings were taken as the correct ones.

Check determinations with the hydrogen against quinhydrone electrode were made on fifteen saliva samples from the same individual and on five samples from four other subjects. Only in a single case, the values by the two methods differed by as much as 0.06 pH unit. The average difference was 0.025 pH unit.

No further experiments were made with the hydrogen electrode since the quinhydrone electrode proved more convenient to use.

*Quinhydrone Electrode Checked against Glass Electrode*—The quinhydrone cells were the same as previously used, except that a small stop-cock was attached at the lower end in order to prevent flow and diffusion within the capillary. The glass electrodes were of the type developed by MacInnes and Dole (4). Fig. 1 shows one of those electrodes supported in a glass vessel, *C*, which contains saliva (or some other solution used in the measurement). *B* is one arm of a U-tube filled with saturated KCl. Suspended in the other arm (not shown) is the reference half-cell (Ag-AgCl electrode in 0.1 *N* HCl). The same salt bridge and reference half-cell were used in the measurements with the quinhydrone electrode.

The measurements were made in a shielded oil bath whose temperature was maintained at 25° ± 0.05°. A Lindemann electrometer served as the null point instrument; the smallest

deflection which could be observed corresponded to 0.5 millivolt. The compensating electromotive force was supplied by a type K potentiometer. Both the quinhydrone electrode and the glass electrode were calibrated against 0.05 M phthalate (pH 3.98<sub>2</sub>) and a phosphate buffer of pH 6.97<sub>4</sub> (measured with the hydrogen electrode).

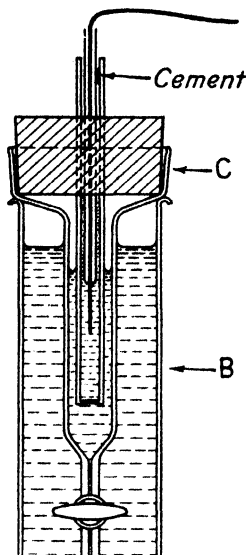


FIG. 1. The glass electrode vessel. C represents the electrode vessel; B, the salt bridge.

The saliva used in the check measurements was thoroughly mixed in the vessel shown on Fig. 2. The electrode vessels were then filled through the capillary attached to its side. Seven check determinations were made. The values obtained by the two methods differed, on the average, by 0.01 pH unit; the largest difference was 0.02 pH unit. The drift observed with the glass electrode was 0.5 millivolt or less; successive readings on samples of the same saliva or buffer solution checked within 0.5 millivolt. The glass electrode was therefore adopted for use in subsequent work.

*pH of Resting and Paraffin-Stimulated Saliva*—The measure-

ments were made in an oil bath at 37° (*cf.* above). The glass electrodes were calibrated against 0.05 M phthalate whose pH was assumed to be the same at 37° as at 25° (5).

The saliva was obtained between 3 and 4 p.m. After a sample of resting saliva (about 8 minutes required) was collected, the subject was asked to rinse his mouth and was given a piece of parawax to chew. In about 3 minutes a sufficient quantity of the stimulated saliva accumulated in the mouth. In order to minimize the loss of CO<sub>2</sub>, the samples were transferred from the mouth to the electrode vessel *C* (Fig. 1) with the aid of a funnel (with a

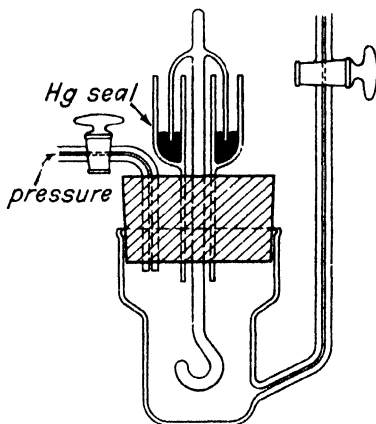


FIG. 2. The mixing cell. The saliva was introduced through the capillary tube attached to the side.

wide cone) which was attached to *C* with a short piece of rubber tubing. With the funnel held between the lips and the teeth, saliva was allowed to flow into the electrode vessel until its level reached 1 cm. above the glass membrane.

Nine samples of resting saliva from nine subjects ranged from 6.37 to 6.89 pH. The pH of nine samples of stimulated saliva, collected immediately after, varied between 6.97 and 7.32. In the case of four of the subjects, additional samples were taken between 11 a.m. and 12 noon and between 5 and 6 p.m. (The subjects had completed their breakfast at 8 a.m., lunch at 12.30 p.m.) The pH values of those samples were 6.40 to 6.88 for resting saliva,

7.00 to 7.27 for stimulated saliva. The average of all of the above results for resting saliva is pH 6.64; for stimulated saliva, 7.13.

*pH Change of Saliva on Addition of Acid and Alkali*—Neither the quinhydrone electrode (in its usual form) nor the glass electrode proved suitable for the potentiometric titration of saliva, probably because of the presence of proteins which precipitated on the electrode surface during the addition of acid. Preliminary experiments with the hydrogen electrode showed that it, too, was

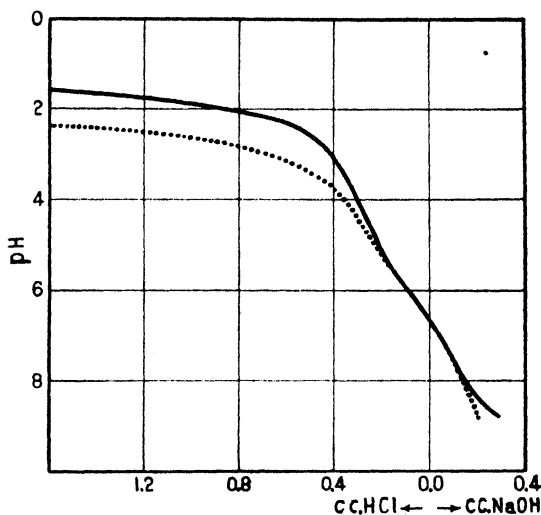


FIG. 3. The titration curves of saliva. The amounts of 0.1  $N$  HCl or NaOH added to a 2 cc. sample of resting saliva are given as the abscissæ. The titration curve of 1:2 saliva is represented by a solid line; that of 1:10 saliva, by a dotted line.

subject to a similar influence. The quinhydrone electrode gave readings reproducible to 0.15 pH unit when used in the titration of dilute (1:10) saliva with 0.1  $N$  HCl. The titration curves thus obtained, however, were nearly identical regardless of the origin of the saliva sample. The curve plotted from the average values for ten samples from ten individuals is shown on Fig. 3.

In the case of more concentrated (1:2) saliva solutions, each titration curve was plotted from pH measurements on a number



of saliva samples from the same individual, but with different amounts of acid or alkali added. The procedure was as follows:

Five men, aged 20 to 45 years, served as the subjects. The saliva was obtained at various hours between 9 a.m. and 9 p.m., but always at least 2 hours after the last meal. Moreover, the time of the day did not vary by more than 1 to 2 hours for a given subject.

The sample was received in a cylindrical funnel which was calibrated to deliver 2 cc. It was introduced, through the side tube

TABLE I  
*pH of Saliva on Addition of Acid and Alkali*

Acid or base added	Amount of acid or base added to 2 cc. saliva	pH (average values)	Difference between maximum and minimum values
	cc.		
0.1 N HCl	1.5	1.61	0.39
	1.0	1.93	0.21
	0.8	2.04	0.65
	0.6	2.32	0.67
	0.5	2.48	1.06
	0.4	3.11	2.51
	0.3	4.05	1.96
	0.25	4.36	2.35
	0.2	5.23	1.04
	0.1	5.94	0.79
0.1 N NaOH	0.0	6.53	0.40
	0.05	7.23	0.71
	0.1	7.64	1.11
	0.15	8.21	0.71
	0.2	8.78	1.51

of the mixing cell (Fig. 2), under a measured quantity of 0.1 N HCl or NaOH made up to 2 cc. with water. The cell contents were stirred, and a portion of the mixture forced up for pH measurement with the glass electrode.

Whenever large amounts of precipitate formed on the addition of acid, measurements were made on two portions from the same sample; the first one consisting of clear liquid and the second containing as much as possible of the precipitate. Such duplicate measurements checked within 0.02 pH unit.

The saliva samples differed markedly in their acid-neutralizing

power. The difference between the lowest and the highest pH reading obtained on the addition of a given amount of acid (or alkali) is found in the fourth column of Table I. The average values for each point on the titration curve are found in the third column. For the curve plotted from those values, one is referred to Fig. 3.

#### DISCUSSION

In beginning a general study of saliva, we were faced with the need for a rapid, precise, and reliable method of pH measurement. The ordinary colorimetric procedure involves dilution with water and the addition of a foreign substance (an indicator). Hence its usefulness is, at best, limited. Besides, the previously published results (1) by the colorimetric and electrometric (hydrogen electrode) methods do not check well enough to recommend either. From the experiments reported above, the MacInnes glass electrode would appear to be reliable to at least 0.02 pH unit. Readings made with two glass electrodes on the same saliva sample usually check within 0.01 pH unit. Some of the electrodes, made half a year ago, have since been used in more than 50 measurements each. The capillary quinhydrone electrode gave results reproducible to 1 millivolt only after considerable practice in its manipulation. It was very sensitive to temperature changes and, occasionally, to other influences which were less easy to trace.

A series of measurements with the glass electrode of the pH of resting and paraffin-stimulated saliva at 37° gave results in approximate agreement with the data found in the literature. The readings obtained with the resting and stimulated saliva form two well separated groups, the pH values of stimulated saliva being higher by an average of 0.5 unit. The range of variation within each group appears smaller than that previously reported, but it may possibly increase when additional subjects become available for study.

None of the electrodes which we tested was suitable for use in a potentiometric titration of saliva. Each one of the titration curves was therefore plotted from pH readings on a series of samples from the same subject, taken in a similar manner and, in so far as was known, under similar conditions. The data thus obtained permit certain conclusions regarding the behavior of saliva as a buffer.

If we assume the pH figures in Table I to represent, at least approximately, hydrogen ion activities, it will be seen that the acid-neutralizing power of saliva decreases markedly between pH 4 and 2.5. On the alkaline side of pH 4, all but a very small fraction of the added hydrogen ion is removed by chemical combination; on the acid side of pH 2.5, more than half of the added hydrogen ion remains uncombined.

This interpretation of the pH data is supported by results obtained by conductometric titration of saliva. Several samples of (1:3) saliva were titrated with 0.1 N HCl. The conductivity increase, at first very slight, became rapid and uniform beyond the point where sufficient acid had been added to obtain pH 4. One may thus conclude that the buffer range of saliva constituents lies, mainly, on the alkaline side of pH 4. Variations in the amounts of these substances would be reflected in relatively small pH changes of saliva as such. On the addition of acid, however, a point would be reached where individual variations of the buffer content of the sample would lead to large pH differences. Further addition of the acid would cause the titration curves of several samples again to approach each other. The figures in the fourth column of Table I (difference between maximum and minimum pH value for every point on the titration curve) demonstrate this effect. In comparing the acid-neutralizing power of several samples of resting saliva, one or two pH measurements on each sample will hence be sufficient provided that suitable amounts of acid have been previously added (for instance, 0.3 or 0.4 cc. of 0.1 N HCl to 2 cc. of saliva).

The ability of saliva to neutralize acids is thought by some to have an important bearing on the etiology of caries. Experiments designed to establish a definite relationship between caries susceptibility and the pH and buffer action of saliva have led to contradictory conclusions. In so far as the lack of agreement among the investigators might be due to their methods of procedure, it is hoped that our results may, in part, contribute to useful knowledge in the field of caries research.

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members of the staff of the New York University College of Dentistry and others who served as the subjects.

#### SUMMARY

The pH values of saliva obtained with the MacInnes glass electrode and capillary quinhydrone electrode differed by less than 0.02 pH unit. The glass electrode gave more reproducible results and proved easier to handle than the quinhydrone electrode.

The average pH values of resting and stimulated saliva, measured with the glass electrode at 37°, differed by 0.5 pH unit.

Saliva acts as a buffer on the alkaline side of pH 4.

Samples of resting saliva vary considerably in their acid-neutralizing power.

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## A STUDY OF THE AVAILABILITY OF *d*- AND *l*-HOMOCYSTINE FOR GROWTH PURPOSES

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It is a rather curious fact that although *d*-cystine cannot be substituted for *l*-cystine in support of growth of animals on a cystine-deficient diet (1, 2), both *d*- and *l*-methionine can replace *l*-cystine equally well under these experimental conditions (3). Since we had found that homocystine could serve in lieu of either methionine or cystine in the promotion of growth of animals on a cystine-deficient diet (4), we became interested in the question of whether both optically active isomers of homocystine were physiologically effective as in the case of methionine or whether specificity in regard to spatial configuration was involved as in the case of cystine.

The homocystine that was used in those experiments was optically inactive, for at that time only the optically inactive material was available. The resolution of homocystine, which has recently been reported from this laboratory (5), has now enabled us to study this question of the utilization of *d*- and *l*-homocystine.

Such a study was of particular interest because of the significance that has been attached to the possibility that homocystine might be an intermediate compound in the metabolism of methionine (4, 6, 7) and that it might be involved in the metabolic relationship that unquestionably exists between methionine and cystine. Other aspects of this theory have already been discussed in considerable detail in a previous communication (8).

If only *l*-homocystine were utilizable, serious doubts would be cast on the theory that homocystine represents the first step in the metabolism of methionine. According to this theory one would expect that both *d*- and *l*-methionine would be converted

in the body to the corresponding homocystine, since, as stated above, both optical isomers of methionine are utilizable for growth

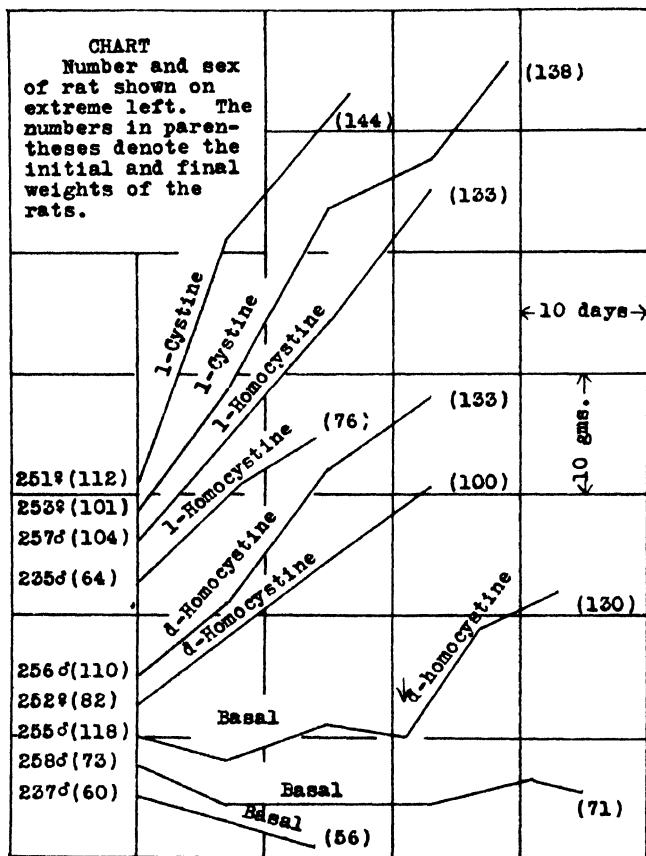


CHART I. Growth curves of rats on a cystine-deficient diet, supplemented with *l*-cystine and *d*- and *l*-homocystine. Rats 235 and 237 were from Litter A; Rats 251 to 253 and 255 to 258, Litter B.

purposes. If this conception be correct, one would expect to find that both *d*- and *l*-homocystine were utilizable.

As shown in Chart I in the experimental part of this paper, both

the dextro and the levo isomers of homocystine are able to support the growth of rats on a cystine-deficient diet. With the amounts of supplements which were ingested, and with this particular basal diet, no notable differences were observed in the rates of growth of the animals receiving *d*- and *l*-homocystine.

## EXPERIMENTAL

Two litters of rats were used for the studies of the availability of homocystine for growth. The basal diet was of the same composition as that employed in the study of the utilization of mesocystine (2) and of pentocystine and homomethionine (8), and

TABLE I  
*Food Consumption*

Rat No. and sex	Days	Supplement	Average daily consumption
			gm.
251 ♀	1-16	<i>l</i> -Cystine	11.4
253 ♀	1-29	"	9.0
257 ♂	1-23	<i>l</i> -Homocystine	9.8
235 ♂	1-14	"	5.5
256 ♂	1-23	<i>d</i> -Homocystine	9.7
252 ♀	1-23	"	7.4
255 ♂	1-21		9.7
	21-33	<i>d</i> -Homocystine	8.5
258 ♂	1-35		6.4
237 ♂	1-14		4.4

consisted of: casein 6.0, dextrin 37.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Osborne and Mendel (9)) 4.0, agar 2.0, and milk vitamin concentrate (Supplee *et al.* (10)) 12.0. It was fed *ad libitum* and a record was kept of the food consumption. All the animals were placed on the basal diet for a fore period of 12 days. At the end of this period, two dextrin pills containing 10 mg. each of *l*-cystine were given daily to Rats 251 and 253. Two pills containing 11.2 mg. of *l*-homocystine, the equivalent in sulfur content of the *l*-cystine supplement, were given daily to Rats 235 and 257, and two pills of 11.2 mg. of *d*-homocystine were given daily as supplements to Rats 252 and 256. Three animals, Rats 237, 255, and 258, were kept as controls on the basal diet alone, or



were given control dextrin pills in addition to the basal diet. After receiving the basal diet for 33 days Rat 255 was fed the *d*-homocystine-supplemented diet.

After the first 3 days of the experimental period the rats refused the homocystine pills, so the supplements were incorporated in the basal diets. For the next period of 10 days the supplements were added to the diets to make final concentrations of 0.2 per cent of *l*-cystine and 0.223 per cent of each of the stereoisomers of homocystine. On the 13th day the concentration of these substances incorporated in the basal diets was increased to 0.3 per cent for *l*-cystine and 0.335 per cent for both *d*- and *l*-homocystine. The average daily food consumption for each animal is given in Table I, and Chart I presents the growth curves.

#### SUMMARY

The present investigation has demonstrated that both *d*- and *l*-homocystine can be utilized for growth purposes by animals on a cystine-deficient diet. This behavior of homocystine with respect to spatial configuration is similar to that of methionine but in contrast to that of cystine. This similarity of homocystine to methionine in the equal availability of its optical enantiomorphs for growth purposes strengthens the conception of the interrelationship in metabolism of homocystine and methionine as discussed above.

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# CALCIUM AND AMMONIUM EXCRETION IN THE URINE OF RABBITS

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(Received for publication, February 9, 1935)

In response to the administration of acid, a definite increase in the ammonium excretion in the urine of most experimental animals is observed (1-4). The rabbit is a notable exception in this respect, as is evident from the data of Winterberg's experiments (5). It was usual in Winterberg's experiments, and in those reported here, to find that in response to acid or ammonium chloride administration, ammonia did not begin to increase appreciably in the urine until 3 or 4 days before the death of the animal, regardless of the length of the experiment or the severity of the acidosis.

Administration of acid to cats (6, 7) results in an increase in the urine of ammonia and fixed base from soft tissue, but almost no increase of calcium excretion.<sup>1</sup>

The experiments of Steenbock, Nelson, and Hart (3) and of Lamb and Evvard (4) on cows and pigs indicated that the calcium of the urine increased with large doses of acid. In comparison to ammonia, it played a minor rôle in neutralization of the acid excreted. Because the animals were fed, it is impossible to tell from their experiments how much, if any, of the calcium increase of the urine was due to increased absorption from the intestinal tract. More recently, balance experiments by Hart, Steenbock, and Kline (8) indicate that increased absorption from the intestines does not account for the increased excretion of calcium in the urine resulting from administration of acid to cows.

<sup>1</sup> That the calcium does not appreciably increase, was confirmed as follows: 30, 45, and 70 cc. of 0.1 N hydrochloric acid per kilo per day respectively were given by stomach tube to each of three fasting cats. The first showed no rise of urinary calcium in a week. The second and third increased 1 mg. of calcium per day. The third cat died in coma on the 4th day.

Man, during the course of prolonged fasts (9-11), excretes in the urine about 5 times as much calcium as is to be expected from the catabolism of his soft tissues. The ammonia excreted simultaneously is sufficient to neutralize 12 to 14 times the amount of acid neutralized by the calcium.

The experiments recorded here show that during the course of a fast, the urinary calcium of rabbits progressively increases, and on increasing the acid excretion by administration of ammonium

TABLE I  
*Urinary Calcium Excretion of Fasting Rabbits*

Day of fast	Rabbit 9, 1.49 kilos		Rabbit 11, 1.90 kilos		Rabbit 12, 1.70 kilos		Rabbit 2, 1.97 kilos	
	pH	Ca	pH	Ca	pH	Ca	pH	Ca
		mg.		mg.		mg.		mg.
1		2.05	6.2	0.26	6.8	0.72	6.6	2.4
2		4.55	6.0	0.83	5.4	5.9*	6.4	8.0*
3		11.8	5.4	5.8	5.2	17.4*	6.0	37.8*
4	6.4	12.6	4.9	7.8†	5.1	46.7*	5.0	31.2
5	5.6	15.3	4.9	8.5†	5.0	83.0		
6	5.1	19.0	4.9	6.2*	5.2	57.6‡		
7		16.0§	4.9	21.2	5.2	41.2‡		
8	5.6	3.3	5.1	5.35¶				

The ammonia excretion per day expressed as mg. of  $\text{NH}_3\text{-N}$  varied as follows: 5.2 to 7.26 (Rabbit 9), 1.5 to 3.5 (Rabbit 11), 1.4 to 3.6 (Rabbit 12).

\* 20 cc. of 0.1 N  $\text{NH}_4\text{Cl}$  were administered (*per os*).

† 40 cc. of 0.1 N  $\text{NH}_4\text{Cl}$  were administered (*per os*).

‡ 44 cc. of 0.1 N  $\text{Na}_2\text{CO}_3$  were administered (subcutaneously).

§ 40 cc. of 0.1 N  $\text{NaHCO}_3$  were administered (*per os*).

|| 60 cc. of 0.1 N  $\text{NaHCO}_3$  were administered (*per os*).

¶ 46 cc. of 0.1 N  $\text{Na}_2\text{CO}_3$  were administered (subcutaneously).

chloride, the calcium of the urine is so far increased as to leave little doubt that part is derived from the skeletal structures.

### Procedure

The animals were confined in metabolism cages. Ammonium chloride was administered in 0.2 N solution (not more than 20 cc. at any one time) by means of a small catheter used as a stomach tube. The pipette and catheter were washed down with not more than 5 cc. of water. Intervals of at least 4 hours were maintained

between repetitions of the dose. Distilled water was kept in the cages in receptacles firmly attached to the bottom.

Before removing the animals from the cages to give them the ammonium chloride, as well as at the end of each day, the bladder was emptied by pressure. Chloroform was kept in the collecting bottles, and each sample was placed in the refrigerator immediately and kept there until the end of the day, at which time the ammonia and pH determinations were made without delay.

Calcium was determined by the method of Fiske and Logan (12); inorganic phosphorus by the method of Fiske and Subbarow (13); ammonia by aerating 10 to 30 cc. for 2 hours, after adding solid sodium carbonate and sodium chloride, and by Nesslerization according to the Folin method for urine (14); total sulfur by the Fiske method (15); and pH colorimetrically by comparison with phthalate and phosphate buffers made according to Clark (16) of samples diluted 1:10 with distilled water. The recorded pH is that obtained after the  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  crystals, present in the urine, had apparently dissolved as a consequence of the dilution. (See below.)

#### DISCUSSION

In collecting urine from cats, it is advantageous to give a large amount of water near the end of each metabolism period to insure that the final sample of urine will be dilute. This procedure was dispensed with after it had caused the termination of several experiments on rabbits. Ammonium chloride instead of the free acid was chosen for the same reason. During an extended fast, rabbits apparently do not tolerate well either acid or large volumes of water in their stomachs.<sup>2</sup>

The samples of urine which contained 1 or 2 mg. of calcium were clear when passed. As the calcium increased, the urines became progressively more turbid when passed, due to the presence of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  crystals. These two factors combined to make the separation of 1 day's output from the next less accurate than can sometimes be attained with cats; but because the bladder was emptied by pressure several times each day, the amount carried over to the next day would probably not represent more than 5 to 10 per cent of the daily output.

<sup>2</sup> Rabbits withstand fasting less than 2 weeks ordinarily.

TABLE II

*Urinary Excretion of Fasting Rabbit 15 to Which  $\text{NH}_4\text{Cl}$  Was Administered*  
Weight, 1.750 kilos at start; 1.285 kilos at end of experiment.

Day of fast	Urine volume	pH	Total S	Creatinine	$\text{NH}_3\text{-N}$	Inorganic P	Ca	0.1 N $\text{NH}_4\text{Cl}$ (per os)
	cc.		mg.	mg.	mg.	mg.	mg.	cc.
1	222	6.8	48.0	89.5		110	1.3	
2*	222	6.4	53.2	100		178	7.2	
3	222	6.4	53.3	101		178	7.3	
4	338	5.6	43.4	64.1	3.4	94	9.8	60
5	260	5.2	54.1	84.0	2.6	128	23.9	60
6	103	5.0	61.4	78.0	2.75	135	60.0	60
7	105	5.0	57.1	65.7	3.84	112	72.3	60
8	130	5.1	54.8	68.0	5.75	111	87.8	60
9	150	5.1	54.1	63.8	5.9	103	103.6	80
10	99	5.0	58.1	64.4	6.7	129	147	80
11	130	5.0	73.1	73.4	19.5	149	191	120
12	114†	5.6	54.3	45.7	34	70.3	94.9	120

\* The amounts of urine excreted during the 2nd and 3rd days were analyzed together.

† Urine for about 18 hours. The rabbit died before the end of the 12th day.

TABLE III

*Urinary Excretion of Fasting Rabbit 19 to Which  $\text{NH}_4\text{Cl}$  Was Administered*  
Weight, 1.880 kilos at start; 1.350 kilos at end of experiment.

Day of fast	Urine volume	pH	Total S	Creatinine	$\text{NH}_3\text{-N}$	Inorganic P	Ca	0.1 N $\text{NH}_4\text{Cl}$ (per os)
	cc.		mg.	mg.	mg.	mg.	mg.	cc.
1	29	6.4	27.1	38		62.7	0.18	
2	52	6.4	45.2	88		83.1	0.60	
3	54	5.8	35.0	77.5	2.8	94.2	13.6	40
4	138	6.0	48.2	146	6.9	149	42.5	80
5	81	5.0	29.2	96	4.9	101	78.8	80
6	127	5.0	31.5	102	2.96	104	112	80
7	70	5.1	29.6	93	3.2	103	113	80
8	65	5.0	31.2	86.5	5.61	117	129	100
9	90	5.1	43.4	105	15.3	105	166	100
10	78	5.3	33.8	81.6	17.4	60	105	100
11								40*
12	60†				18.7			

\* Diarrhea, urine contaminated.

† Diarrhea, urine dark, probably contaminated. The rabbit died at the end of the 12th day.

Creatinine and total nitrogen determinations (or total sulfur when ammonium chloride was administered) were performed to detect possible changes in the rate of tissue catabolism which might conceivably have a bearing on the rate of calcium excretion. From the rate of excretion of these products, no marked change in the rate of catabolism was indicated during the course of the experiments, so to conserve space, the creatinine and sulfur determinations are given only for Rabbits 15 and 19.

Previous to the tests, Rabbits 2 and 11 were fed 5 and 3 days respectively on corn and apples (*ad libitum*). The others had previously subsisted on green food and grains (*ad libitum*). If they had previously eaten a food of high acid value, it would not have been surprising to find an excretion of considerable calcium on the 1st day of the fast. None was encountered, owing no doubt to the fact that rabbits do not by choice consume an acidotic diet.

### Results

In response to the excretion of acid products derived only from the use of their own body tissues, the urinary calcium of two rabbits increased progressively from 0.97 and 2.05 mg. of calcium on the 1st day, to 15.1 and 19.0 mg. on the 7th and 6th days respectively.

The results of three fasts (4 to 8 days duration), during which small amounts (40 to 100 cc. of 0.1 N ammonium chloride) were administered, indicated an individual difference with regard to the promptness with which urinary calcium excretion increased in response to ammonium chloride administration. Rabbit 11 reacted by excreting highly acid urine (pH 4.9) for 3 days before the calcium rose to 21 mg. per day. With Rabbits 2 and 12, the calcium excretion was 37.8 and 46.7 mg. per day respectively before the urine reached pH 5.0. Administration of sodium bicarbonate *per os* (Rabbit 9) or sodium carbonate subcutaneously (Rabbits 11 and 12) decreased calcium excretion. Administration of base could not, however, be continued for sufficient time to cause a decrease of the calcium to its original level.

The influence of ammonium chloride administration on the urinary calcium excretion is more clearly indicated with longer fasts and greater ammonium chloride administration. Rabbits 15 and 19 were fasted 12 days, during which a total of 700 cc. of

0.1 N ammonium chloride was given to each. In the first case the calcium increased progressively from 1.3 mg. on the 1st day to 191 mg. on the 11th. In the second case, the increase was from 0.18 mg. on the 1st day to 166 mg. on the 9th. The total amounts of calcium excreted during the periods were 806.1 mg. of calcium (Rabbit 15), and 760.7 mg. of calcium (Rabbit 19) (403 and 381 cc. of 0.1 N calcium, respectively).

This is at least 10 times the amount to be expected from soft tissues as estimated from the loss in weight, or 3 times as much as would be expected to be contained in the soft tissue in the beginning of the experiment. The results give no indication that phosphate excretion was simultaneously increased. The inorganic phosphate in rabbit bone is stoichiometrically equivalent to 90 to 95 per cent of the calcium. If the phosphate excretion had been increased to correspond with the phosphate content of bone, an extra 50 mg. of phosphorus or more could have been expected near the end of these experiments. The ammonia excretion was insignificant (2.6 to 6.9 mg. of  $\text{NH}_3\text{-N}$ ) until 4 days before the death of one rabbit, and 2 days in the case of the other. On the 11th day, with Rabbit 15, and on the 10th day with Rabbit 19, the acid neutralized by ammonia was respectively 14.6 and 23.6 per cent of that neutralized by calcium (approximately 7 and 12 per cent of the total). No attempt is made from these experiments to make an accurate comparison between quantity of calcium excreted and quantity of acid eliminated, because it is assumed (*a priori*) from experience with other animals (6) that the amount of fixed base from soft tissues available for neutralizing urinary acidity is variable. The urinary calcium then could be expected to comprise varying quantities of the total base of the urine just as ammonia comprises varying quantities of the total base during short periods of acid régime with those animals which use ammonia primarily to neutralize urinary acidity. The case with urinary calcium excretion in rabbits has the additional complication that a variable supply of calcium (from bone) may be available. Near the end of the experiments with Rabbits 15 and 19, approximately 50 per cent of the total acid was neutralized by calcium.

It is evident that cats and rabbits represent opposite extremes in regard to the excretion of calcium in the urine in response to the administration of acid. It appears not unreasonable to suggest

that the continued excretion of calcium and ammonia in the urine of man during fasting indicates that he represents an intermediate type.

#### SUMMARY

The urinary calcium of fasting rabbits, unlike that of cats, increases in response to excretion of acid in the urine.

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# **OBSERVATIONS ON THE CHEMICAL NATURE OF A HEMATOPOIETIC SUBSTANCE OCCURRING IN LIVER**

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The object of the following communication is to describe a product isolated from liver extract which appears to possess interest partly on account of its chemical make-up and partly on account of its relationship to the constituents in liver which Minot and his coworkers have shown to be effective in causing blood regeneration in pernicious anemia. It may at once be emphasized that the material to be described has not been crystallized and such crystalline derivatives as have been obtained are of little value in establishing strict chemical individuality; in fact, the available evidence is against the view that we are dealing with a single substance. On the other hand, relative constancy of composition, the isolation of crystalline products of hydrolysis, and the apparent fact that clinical potency is consistently associated with the compound and is absent from preparations in which it has been either removed or chemically altered, would seem to justify its claim to consideration.

Progress in the chemical investigation of the hematopoietic substance or substances in liver has been curiously slow and discouraging. Two reasons are largely responsible for this: first, that bitter experience has shown that most of the chemical methods of choice result in inactivation of the product, and, secondly, the great scarcity of suitable clinical cases essential for testing the activity of isolated products. For these reasons it seemed wise to record our results at the present time, leaving obvious extensions of the work to the future and so to give other workers an early opportunity to test the validity of our conclusions. Our observa-

tions, moreover, give obvious indications of improved methods of preparation of material for routine clinical use.

The isolation of the substances dealt with in this paper depends essentially on the following considerations. First of all, it was found that the clinically active material in liver extract was completely, or nearly so, precipitable by Reinecke salt in acid solution. If an amount of roughly purified liver extract equivalent to two clinical doses is precipitated by this reagent, the filtrate is completely clinically inactive, while the precipitate on regeneration is fully active (Cases 392148, 364172). The decomposition of the Reineckate precipitate presented a problem requiring special methods which we believe to be solved successfully. The use of metallic salts was excluded, since these resulted in clinical inactivation, while the direct use of solvents was found impracticable on any considerable scale and involved serious losses of material. The separation of the bulk of the Reinecke acid from a weak alcoholic solution of the precipitate as the sparingly soluble salt of a tertiary base such as dimethylaniline gave satisfactory results, as described in the experimental portion of this paper. The small amount of unprecipitated Reinecke acid was removed with solvents such as amyl alcohol. It will be noted that these procedures are carried out in virtually neutral solution, a consideration of great importance. Next it was found that a great deal of preliminary purification of commercial liver extract could be effected by precipitation with calcium acetate or chloride in 75 to 80 per cent alcoholic solution. The bulk of the clinically active material was found in the filtrate, while the precipitate on regeneration gave considerable amounts of material precipitable by Reinecke acid which was clinically inactive (Case 415857). It was clear therefore that a preliminary purification with the aid of calcium acetate was a desirable step to incorporate prior to precipitation of the potent material with Reinecke acid.

At this point it may be well to emphasize the significance attached in this paper to the terms "active" and "inactive" as applied to clinical tests. Having learned by experience that 100 mg. or less of a suitably purified product were capable of producing a maximum reticulocyte response under favorable clinical conditions, we have regarded as "inactive" products which gave no significant change in reticulocytes when given to a suitable case

in doses of 100 mg. or more. It will be seen therefore that the term "inactive" does not mean of necessity that the material was quantitatively free of active substance but rather that in doses of 100 mg. or more the proportion of active material was too small to produce demonstrable blood changes.

The clinically potent material recovered by precipitation with Reinecke acid after removal of substances precipitable with alcoholic calcium acetate was separated by means of strong alcohol from certain well known tissue components such as choline and traces of creatinine. The amount of purification that could be thus effected was limited and better success lay in other directions. It was found that the product could easily be separated into two fractions, one precipitable from neutral or feebly acid aqueous solution by saturation with ammonium sulfate and the other remaining in the filtrate. The material precipitable by ammonium sulfate contained the clinically active material (Case 417195), while comparable amounts of the substances in the filtrate were inactive (Case 417195). It was found that the material precipitated by ammonium sulfate could be redissolved in water and again precipitated with ammonium sulfate, and the whole process repeated several times without loss of potency (Cases 356184, 316263, 419573, 423439). It should be stated at this point that incentive to use ammonium sulfate for the present purpose is really due to Dr. F. H. Carr who several years ago expressed to one of us (H. D. D.) his belief that the clinically active material in liver was precipitable by this salt in strongly acid solution.

The further purification of the product salted out by ammonium sulfate presents serious difficulties. Precipitation as gold or silver salts resulted in complete inactivation as previous experience would lead us to expect. Precipitation with picric acid gave an oily picrate which was clinically active (Case B. H.) but very little chemical separation was effected by this reagent. Flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) precipitated between 65 and 75 per cent of the product in the form of an oily substance which apparently was somewhat unstable. This procedure resulted in very definite chemical separation, for the material recovered from the filtrate was completely inactive clinically and was chemically sharply different from the material in the precipitate. Thus, on hydrolysis of the material from the filtrate hexone

bases were virtually absent. Unfortunately the recovery unchanged of the active material from the flavianic acid precipitates is not an easy matter and ordinarily requires decomposition with a concentration of mineral acid, which is decidedly disadvantageous. There is no question, however, of the clinical activity of the material precipitated by flavianic acid as shown by Case 76032. 80 mg. of material from the flavianic acid filtrate gave practically no response. Attempts to devise more gentle methods of decomposing the flavianate have not proved entirely successful. Furthermore, we have observed the fact that flavianic acid is a very imperfect precipitant for the active material unless the latter has been subjected to extensive prior purification. In view of these findings we abandoned the use of flavianic acid.

More encouraging results were obtained following the observation that the material salted out by ammonium sulfate could be fractionated by the use of magnesium sulfate. A much higher concentration of the latter salt is necessary for "salting out" than with ammonium sulfate. On taking the material that has already been precipitated once or twice by ammonium sulfate, it was found that about 75 per cent was precipitable on complete saturation with magnesium sulfate; while on repeating the process by dissolving in water and again saturating with magnesium sulfate, only about 3 per cent was found in the mother liquor, thus indicating a definite separation of the components of the original product. As will be seen later, some definite differences are observable in the products of hydrolysis of the products soluble and insoluble in magnesium sulfate solution respectively.

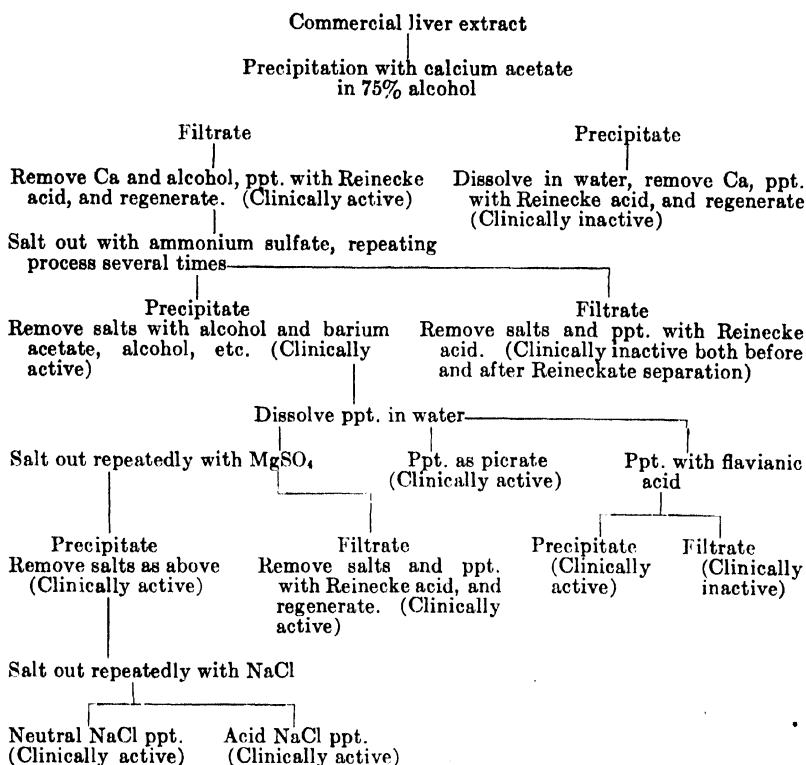
On recovering the material repeatedly precipitated by magnesium sulfate and removing inorganic salts by appropriate methods indicated in the experimental section of this paper, it was found that the product was consistently clinically active (Cases 297466, 81298, 71632, 237059, 382735). It will be noted that in two cases 30 mg. and 50 mg. gave a barely perceptible rise in the reticulocyte count, while 80 mg. gave a maximal response. This material at the present time represents our most highly purified clinically active product. On the other hand, the material in the magnesium sulfate mother liquors was separated from the bulk of inorganic salts by means of alcohol, and the solution concentrated to remove alcohol and again precipitated with Reinecke salt. On

decomposing the Reineckate, the product, which is undoubtedly a mixture, was found to have some slight but definite clinical activity (Cases 426486, 382735). Whether this result is due to incomplete separation of the magnesium sulfate-precipitable material or to the presence of other active products cannot at present be assumed definitely, though the probability or even certainty of the magnesium sulfate filtrates containing some of the active material, the bulk of which is precipitable by magnesium sulfate, cannot be questioned. Since the above was written, we have succeeded in further fractionating the  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{MgSO}_4$  preparations with the aid of sodium chloride. We find that about 43 per cent of the material may be separated by salting out in essentially neutral saturated solution, while an additional 50 per cent is precipitated from the filtrate on adding hydrochloric acid to 0.2 N concentration. In each case, the precipitations were repeated under similar conditions. A small residue, about 7 per cent, remained in the filtrate and is recoverable with ammonium sulfate. The composition and specific rotation of these "neutral sodium chloride" and "acid sodium chloride" preparations are very similar to each other and to the material from which they are prepared. We have been able to test clinically the neutral salt preparation and have obtained a good reticulocyte response (Case 442154). A single clinical test of the "acid sodium chloride" fraction (180 mg.) showed definite activity though the response was not maximal. The red blood cells rose from 1.7 to 2.4 millions and the reticulocytes rose from 1 to 16 per cent. A decision as to whether, as appears possible, the neutral salt fraction is actually more potent than the acid salt fraction must await opportunity for further clinical tests.

The general scheme of separation, omitting details, is summarized in the following diagram.

The clinically potent products prepared as above indicated were obtained as light buff-colored granular powders after precipitation, first of all, with alcohol and ether and later with absolute alcohol. They are all extremely soluble in water, have a neutral reaction to litmus, and are soluble in dilute alcohol.

On hydrolyzing the relatively crude material salted out once with ammonium sulfate, convincing evidence was obtained of the presence of the following compounds: an aminohexose, histidine,



arginine, lysine, aspartic, glutamic, and other dibasic amino acids, hydroxyproline, leucine, glycine, and possibly traces of phenylalanine and proline. The phenylalanine and proline, which are only present in quite small amount, disappear in the later stages of purification, while in the best purified product obtained by repeated salting out with magnesium sulfate the histidine is reduced to less than 0.1 per cent and the amount of glutamic acid is so slight that it could not be separated either as hydrochloride or zinc salt. The amount of glycine is also apparently diminished.

By the use of methods partly based on actual separations and partly by analytical procedures which will be referred to in the experimental section we believe that the yield of products of hydrolysis of our best purified preparations is approximately as follows:

histidine trace, less than 0.1 per cent, arginine 14 per cent, lysine 5 per cent, leucine 15 per cent, hydroxyproline 10 to 14 per cent, a monoaminodicarboxylic acid fraction separated as calcium salts, 41 to 45 per cent, of which almost half can be isolated as aspartic acid, while glycine approximates 5 per cent. The amount of aminohexose we believe to be at least 15 per cent, based on colorimetric and other estimations but not on actual isolation. The following tabulation gives some idea of the composition of four of the clinically active products referred to.

Product I. This represents products purified through alcoholic calcium acetate, one or more precipitations with Reinecke acid, and repeated salting out with ammonium sulfate.

Product II. Represents the products obtained from Product I by repeated salting out with magnesium sulfate.

Product III. Represents the material recovered from the magnesium sulfate filtrates.

Product IV. Represents the product obtained from Product I by precipitation with flavianic acid.

Product No. ....	I	II	III	IV
Carbon .....	49.8-50.3	50.0-51.4	49.9-50.5	49.4
Hydrogen .....	7.2- 7.4	7.0- 7.2	7.3	7.0
Nitrogen .....	14.6-15.0	15.2-15.4	15.7	15.1
NH <sub>2</sub> -N (Van Slyke) .....	0.7- 0.9	0.4- 0.5	0.5- 0.6	0.8
NH <sub>2</sub> -N after hydrolysis .....	10.0-10.2	10.6-10.8	10.6-10.9	9.8-10.1
Specific rotation, degrees .....	-88 to -94	-95 to -106	-101	-78 to -85

An inspection of the above figures shows an approximation to constancy of composition which is perhaps more apparent than real. It is noteworthy that Product II, which we are inclined to regard with most favor, shows the lowest amino nitrogen before hydrolysis and the highest specific rotation. In the subsequent discussion it is assumed, unless otherwise stated, that the substance referred to is of similar origin and preparation.

The yield of material from commercial liver extract is approximately as follows: After preliminary purification as described, the crude Reinecke acid precipitate gives about 2 to 2.5 per cent yield of substance, of which about half (1 to 1.25 per cent) is salted out



by ammonium sulfate. Of this latter product, about three-quarters (0.75 to 0.9 per cent) may be recovered either as flavianate or by salting out repeatedly with magnesium sulfate. The actual amount present is naturally materially greater.

In spite of the variety of amino acids obtained on hydrolysis, the substance does not give a typical biuret reaction. On adding dilute copper sulfate to a solution made alkaline with sodium hydroxide in the ordinary way, a yellow color suggestive of reduction is obtained and on prolonged boiling a little cuprous oxide may settle out. If the order is reversed and copper sulfate is added first, a green color is produced which turns yellowish red on adding sodium hydroxide. If, however, a relatively large excess of copper sulfate is added to a very dilute solution of the substance, a feeble biuret reaction may be observed on adding sodium hydroxide and the color increases somewhat on standing. The cause of the abnormal reaction is probably connected with the presence of glucosamine. If the substance is heated for half an hour on the steam bath with normal sulfuric acid, the sulfuric acid then removed with barium carbonate, and the filtrate evaporated to dryness and extracted with hot alcohol, it will be found that the residue gives a moderate biuret reaction. Ammoniacal silver solutions are slowly reduced on boiling; while after preliminary heating for a few minutes with dilute mineral acid, both ammoniacal silver and Fehling's solution are freely reduced.

While it is naturally unsafe to assert the absence of other amino acids than those previously enumerated in the complicated mixture resulting from hydrolysis, yet repeated examination has so far failed to reveal other substances. The complete absence of pyrimidine, and purine bases, and of pentoses and desoxyglucose was repeatedly established. The substance does, however, give a definite though not intense Molisch reaction with  $\alpha$ -naphthol, and at first this was a source of anxiety as a possible indication of some other sugar complex than glucosamine. However, it was found that glycyglucosamine, alanylglucosamine anhydride, as well as partially hydrolyzed chitin, all gave good positive Molisch reactions, even though glucosamine itself gives no reaction, while N-acetylglucosamine gives a bare suggestion of a positive reaction. The positive Molisch reaction in the substance from liver is therefore not surprising. The aminohexose complex in our substance is

not acetylated, for it yields no acetic acid on hydrolysis, nor does it give the dimethylaminobenzaldehyde reaction for acetylglucosamine. Certain decomposition products, particularly those derived by inactivation with alkali, may give the dimethylaminobenzaldehyde reaction for acetylglucosamine, but we have good reason to believe that this reaction is in no sense specific. The absence of sugar groupings other than those of the aminohexose type is indicated by negative reactions with phloroglucinol and naphthoresorcinol and the fact that only a bare trace of color is developed with the Tillmans-Philippi (1) reaction with orcinol and sulfuric acid or the similar reaction of Dische and Popper with indole and sulfuric acid. These tests are extraordinarily delicate and give a strong color reaction with the greatest variety of carbohydrates and allied substances other than glucosamine. The absence of carbohydrate groupings other than an aminohexose is of special importance in view of the fact that up to the present we have not been able to isolate a satisfactory specimen of glucosamine hydrochloride in spite of a number of attempts with various methods. The evidence for glucosamine is therefore of an indirect kind, and would apply equally well to isoglucosamine or fructose-azine. It is based, first of all, on the easy formation of remarkably pure phenylglucosazone when the substance is heated on the steam bath with phenylhydrazine in 5 per cent acetic acid. Chondrosamine, isomeric with glucosamine, gives the osazone of galactose under similar conditions, which is easily distinguished from glucosazone by lower melting point and differing solubility. Secondly, if the substance is partially hydrolyzed with *N* hydrochloric acid and then warmed with acetylacetone in alkaline solution according to the method developed recently by Elson and Morgan (2) for the quantitative estimation of glucosamine, pyrrole derivatives are formed which can be estimated colorimetrically with *p*-dimethylaminobenzaldehyde. Lastly, we have observed that glucosamine yields about 90 per cent of its nitrogen as ammonia on distillation with dilute sodium hydroxide, so that by estimating the total yield of ammonia on distilling our substance with alkali and making suitable correction for the ammonia derivable from arginine, it was possible to get an indirect estimation of glucosamine which checked reasonably well with the colorimetric estimation. (It may be noted in passing that Ledderhose regarded

### Hematopoietic Substance in Liver

The fact that the compound from liver does not give a typical biuret reaction and contains remarkably little amino nitrogen, the whole of which may be referred to the terminal  $\epsilon$ -amino of the lysine group, and the apparent absence of any reducing (aldehyde) group, together with the presence of many hydroxyl groups capable of acetylation or benzoylation would seem to exclude the first and third types of glucosamine peptide and make the anhydride type of linkage the more probable one. Whether the aminohexose groups in our substance are united in polysaccharide form, such as the chitobiose obtained from chitin by Bergmann, Zervas, and Silberkweit (7), remains unsolved. Preliminary experiments on the "acetolysis" of the substance have given unsatisfactory results, for, while an acetylated compound readily soluble in chloroform is obtained, there are still amino acid groups attached to the glucosamine skeleton. The fact that our substance is not precipitated by trichloroacetic acid, ferrocyanic acid, or rufanic acid but is precipitated by tannic acid would tend to allocate the compound rather to the peptone than the albumose group. On the other hand, its easy separation on salting out with ammonium sulfate might be regarded according to convention as indicative of an albumose character, but, since synthetic peptides are known containing only three or four amino acids which can be salted out, it would seem that the distinction is a purely arbitrary one. On boiling with sodium carbonate and picric acid according to the Abderhalden procedure, a fine red color is produced which might be taken as indicative of diketopiperazine groups, but, since glucosamine gives precisely the same reaction, it would seem that the test is without value in the present circumstances. All of our preparations, even when virtually free from histidine, give a positive orange-red diazo Pauly reaction. We believe this to be associated with the aminohexose grouping. For, while pure glycyglucosamine hydrochloride gives no reaction, it gives a positive test if previously evaporated with barium carbonate. Alanylglucosamine anhydride also gives a positive reaction.

Brief reference may be made as to the possible molecular weight of our product. It will be recalled that Fischer found that the cryoscopic method gave good results with the simpler peptides, while for tetra- and hexapeptides the results were only about 20 per cent too low. On the other hand, leucylhexaglycylglycine

gave "schwankende und ganz unwahrscheinliche Werte." Three preparations corresponding to those previously designated as Products I, II, and IV gave the following results.

Preparation No.	C	$\Delta$	Mol. wt.
I	5.0	0.200	475
I	5.0	0.215	442
II	6.0	0.230	496
IV	6.0	0.223	511

Preparation IV was practically ash-free, while the other specimens contained about 0.5 per cent of inorganic impurities. It is obvious that these results must be far too low for a substance which could yield the variety of products of hydrolysis that we have identified. In discarding these observations it may be recalled that Paal and also Ciamician and Zanetti obtained by this method equally improbably low results for a variety of albumoses and peptones, and Goto's estimation of the molecular weight of protone from protamine, namely 423, is equally improbable.

A much higher estimate of the molecular weight may be derived from observations on the titratable acidity of our products in 80 per cent alcohol with phenolphthalein as indicator. It was found that 1 cc. of N sodium hydroxide was equivalent to 1.10, 1.53, 1.43, and 1.66 gm. respectively of the four products previously referred to. This corresponds to an average molecular weight of some multiple of 1430, according to whether one or more carboxyl group is present in the molecule. The addition of formaldehyde makes practically no difference to the titration values, although the possibility of a carboxyl group masked by the guanidine group of arginine must be considered. It must be borne in mind that the experimental error in such titrations of substances of high molecular weight, which are difficult to obtain free from every trace of inorganic impurity, is of necessity large. An independent estimate based on the assumption that 1 molecule of lysine (5.0 per cent) is present in the product would give an indicated molecular weight of about 2920. The analysis of the Reineckate indicates a preparation containing about 36 per cent Reinecke acid, from which a molecular weight of some multiple of 570 may be calculated.

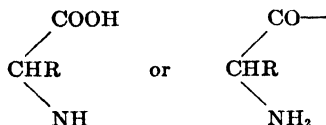
The action of enzymes on our product was obviously of interest

in connection with the great disparity known to exist in the therapeutic effect of liver extract given by mouth and parenterally. We find that the action of pepsin in 0.1 N hydrochloric acid is very slow although perceptible. After 14 days the increase in amino nitrogen is only about 8 or 9 per cent of the total nitrogen. In accord with these findings we record a single experiment in which a large dose of material (200 mg.) had been digested with half its weight of pepsin for 5 days but still was able to produce a marked reticulocyte response (Case 433695). Glucosamine is not liberated during peptic digestion. Pure pancreatic juice appeared to be without action, while erepsin brought about a slow but almost complete hydrolysis. In this connection it is desirable to record an experiment in which a commercial preparation of autolyzed liver was allowed to stand at room temperature in the presence of chloroform for about 2 months. On regenerating the material precipitated by Reinecke acid, it was found that only the barest trace could be salted out with ammonium sulfate and the material was clinically inactive (Case 354091). It seems reasonable to conclude that prolonged autolysis had decomposed the originally active product.

It has already been stated that the clinical activity of the product is readily abolished by the action of alkali. 24 hours exposure to 0.5 N sodium hydroxide at room temperature completely inactivates the substance, and undoubtedly much less drastic conditions would be equally effective. For example, if a concentrated aqueous solution of our substance is made alkaline to phenolphthalein by the addition of concentrated barium hydroxide solution and then precipitated with alcohol, it is found that the precipitated barium salt on decomposition gives a product usually clinically inactive (Case 389711 and others). It is significant that the inactivating effect on clinical potency of cold alkali, as well as short boiling with N sulfuric acid, is precisely what might be anticipated from a substance of glucosamine-peptide nature. The action of alkali on the substance involves extensive racemization of amino acid groups as well as a marked reduction in the specific rotation of the reaction product. In one such experiment in which the product was exposed to the action of N sodium hydroxide for 24 hours it was found on subsequent hydrolysis that practically complete racemization of the arginine, lysine, and leucine groups

had taken place. It is therefore concluded that the  $\alpha$ -amino and adjacent carboxyl groups of each of these acids are substituted

or in other words they are "internally bound" in the  $\begin{array}{c} \text{CO—} \\ | \\ \text{CHR} \\ | \\ \text{NH—} \end{array}$  form and not as



The question of the activity of the dibasic acids is complicated by the fact that aspartic acid is racemized during the process of acid hydrolysis to a much greater extent than is usually supposed. Some optical activity still persisted in the dibasic acid fraction. Although the ammonium sulfate mother liquors remaining after the salting out of the clinically active product contained a great deal of aminohexose in combination, and were found to be clinically inactive (Case 316263), it appeared worth while to test the effect of large doses of glucosamine hydrochloride and of N-acetylglucosamine on pernicious anemia patients. It was found that doses of from 2 to 4 gm. of these substances given parenterally were completely inactive and failed to influence the reticulocyte count in the slightest degree. Perhaps at this point we may be permitted a purely speculative digression. Some 3 years ago we (8) described a tribasic acid present in liver which was convertible into pyrrole derivatives and which we regarded as an intermediary metabolite in the synthesis of blood pigment. In considering the origin of this substance we considered the possibility of its being derived in part from the "oxidation of some substance containing the leucine skeleton such as, for example, leucyl- $\beta$ -alanine." While special search among the products of hydrolysis of our substance has completely failed to detect  $\beta$ -alanine, the presence of large amounts of aspartic acid, which only differs from  $\beta$ -alanine by 1 molecule of carbon dioxide, and of leucine, might possibly prove to be significant in relation to the ultimate understanding of blood pigment synthesis *in vivo*.

It is obvious that a substance of the chemical nature of that

under discussion might well be considered as having intimate relations with the rather indefinite group of substances classified as mucins, although, unlike the latter, our products contain neither acetic, sulfuric, nor glucuronic acid groups. In addition to experiments aiming at a more rigid purification of our product, we are at present engaged in a study of the products of enzyme action on mucins of various origins. The possible relationship of our substance to products of the partial digestion of gastric mucin is obvious and is of interest in connection with the therapeutic effects of dried gastric tissue (ventriculin) and of concentrated gastric juice. It may also be mentioned that therapeutic doses of our substance produce a definite though small increase in the reticulocyte count of some normal individuals probably similar to that observed by Jacobson in guinea pigs. We are not reporting these experiments at this time, but prefer to wait until further data are available.

In conclusion, reference must be made to the fact that, apart from the solubility properties of our preparations (*i.e.* soluble in water and diluted alcohol, insoluble in absolute alcohol and ether), there is almost no point of agreement between the properties of the purified preparations described by Cohn, McMeekin, and Minot (9) and our own. For example, their active material gave no Molisch reaction or diacetyl reaction, was free of proteins and carbohydrates, appeared to be free of arginine, and gave no precipitate with picric, picrolonic, and flavianic acids. They concluded that "the active principle effective in pernicious anemia is not an amino acid but a nitrogenous base, the nitrogen of which exists as in a secondary or tertiary amine." It is hard to reconcile their conclusions with our own findings. One obvious explanation might be sought in the presence of more than one type of hematopoietic substance in liver but our own experiments lend no support to such an interpretation. Further investigation is clearly necessary.

The responsibility for the whole of the clinical tests lies with one of the authors (R. W.) while the other (H. D. D.) is responsible for the larger part of the chemical work.

#### EXPERIMENTAL

In most of our experiments we have made use of commercial liver extract as starting material and we are indebted to Eli Lilly



and Company for generous supplies of their No. 343 preparation. The following description may be regarded as typical of a large number of preparations which have varied but slightly in detail and sequence of operation.

Liver extract powder (500 gm.) is suspended in 500 cc. of warm water (50°) and vigorously stirred to avoid formation of lumps. Calcium acetate or calcium chloride (50 gm.) is then added to the uniform but turbid mixture and mechanical stirring is continued. When the salt is dissolved, 2 liters of 95 per cent alcohol are added gradually with continued stirring, and then the mixture is allowed to settle. A thick, bulky precipitate settles out and is removed by filtration or decantation, the residue being washed with 80 per cent alcohol and then discarded. The precipitate contains among other substances a variety of peptides. On regenerating the precipitate, by solution in water, removal of calcium as oxalate, isolation of the material precipitable by Reinecke acid, and submitting it to biological testing, it appears that less than 10 per cent of the clinically active material is lost in this preliminary operation.

Ammonium oxalate solution is added by degrees to the alcoholic filtrate from the calcium precipitation until the whole of the calcium is precipitated and the calcium oxalate is removed by combined decantation and filtration. The filtrate is then concentrated under reduced pressure in order to remove the alcohol. The residue is diluted to about 700 cc., cooled thoroughly, and then made just acid to Congo red by the addition of sulfuric acid. A 10 per cent solution of Reinecke salt in warm water (50–60°) is then added with vigorous stirring. About 30 gm. of the salt will be required for complete precipitation. If necessary, a little more sulfuric acid is added so as to maintain a faint acid reaction to Congo red, and then the whole is placed in a cool place (10°) overnight. Extreme refrigeration is not desirable as it leads to the separation of some gummy material which slowly redissolves as the temperature rises during filtration. The granular precipitate is filtered with suction on a large Buchner funnel, washed with ice water, and then sucked as dry as possible, the spatula being used freely. This is a somewhat tedious but very necessary operation. The precipitate is then transferred to a wide mouthed bottle and shaken with methyl alcohol so as to break up the lumps of the

precipitate. The amount of methyl alcohol to be used requires some judgment, since too little will lead to incomplete decomposition of the precipitate, while too much interferes with the removal of the Reinecke complex. In general the aim should be to arrive at a final concentration of 35 to 40 per cent of methyl alcohol in the mixture. In the present case, with a well drained precipitate not more than 100 cc. of methyl alcohol should be necessary. The mixture is then heated in a water bath to about 50°. Most of the precipitate will have dissolved, but some sparingly soluble material such as choline Reineckate will remain in suspension. Without filtering, an excess of dimethylaniline (20 cc.) is then added and the mixture shaken on a machine for at least half an hour. It is then diluted with half its volume of water and placed in a refrigerator overnight. The bulk of the Reinecke complex is precipitated as the finely crystalline dimethylaniline salt and is removed by filtration. The brown filtrate is concentrated under reduced pressure to remove methyl alcohol and excess dimethylaniline and the remaining Reinecke acid is removed by repeated extraction with amyl alcohol. Usually four or five extractions are sufficient. The residual aqueous solution, which has a distinctly acid reaction, is neutralized to litmus by the cautious addition of ammonium carbonate solution and then concentrated under diminished pressure to a volume of about 100 cc. The clear cold solution is vigorously stirred while an amount of powdered ammonium sulfate sufficient for three-quarters or more saturation is gradually added. Precipitation begins when almost half the ammonium sulfate is added. The sticky brown precipitate is washed by decantation as far as possible, with saturated ammonium sulfate, and then well drained. The precipitate is then dissolved in a smaller amount of water, *e.g.* 50 cc., and the precipitation with ammonium sulfate repeated as often as desired. If it be desired to separate the material at this stage, corresponding to Product I referred to in the introduction, the precipitate is dissolved in a minimum of cold water with stirring and the bulk of the adhering ammonium sulfate is precipitated by the addition of 3 volumes of alcohol followed by refrigeration. The residual sulfate in the filtrate is then quantitatively removed with barium acetate. The filtrate from the barium sulfate after treatment with decolorizing charcoal is then concentrated to a thick syrup under diminished pressure.

The residue is then mixed with 40 to 50 cc. of methyl alcohol which is added by degrees. While the dry substance is not dissolved appreciably by anhydrous methyl alcohol, the amount of water in the syrup is sufficient to keep the desired product in solution. The methyl alcoholic solution is kept at a low temperature for some hours so as to allow an opportunity for the separation of any minute amount of inorganic or other impurity. The clear alcoholic solution is then filtered into 3 volumes of dry ether, when the product separates out as an amber-colored mass. The ether is then replaced by a mixture of absolute alcohol and ether 1:1 and finally by absolute alcohol alone. The buff-colored granular mass is filtered off, washed with alcohol, and at once dried *in vacuo*. For analytical purposes the product was redissolved in a little water and reprecipitated with alcohol and ether. The dry powder is not particularly hygroscopic, but when moistened with solvents such as alcohol and ether the latter may absorb enough moisture from the air to cause some deliquescence of the substance on prolonged exposure. For purposes of analysis the substance was dried at 60° over phosphorus pentoxide. The following typical results are corrected for a minute amount of ash.

Sample 433-B.	C 49.8,	H 7.2,	N 14.9
" 271.	" 49.9,	" 7.3,	" 15.2
" 421.	" 50.3,	" 7.3,	" 14.7

The specific rotation was observed in aqueous solution. Distinct preparations gave the following values:

$$\begin{aligned}\alpha &= -3.21^\circ, c = 3.60, l = 1.0, [\alpha]_D^{20} = -89^\circ \\ \alpha &= -3.30^\circ, " = 1.70, " = 2.2, [\alpha]_D^{20} = -88^\circ \\ \alpha &= -1.88^\circ, " = 2.0, " = 1.0, [\alpha]_D^{20} = -94^\circ\end{aligned}$$

The properties of the product have been sufficiently described in the introduction so that repetition is unnecessary. The final removal of inorganic salts from the salted out material is not entirely easy, and, when in some experiments we attempted to remove ammonium sulfate by distillation at low temperature and pressure with precipitated barium carbonate, we have the impression that the product was less clinically active. In fact, in general we have the impression, but on quite insufficient evidence, that the final steps in the preparation of ash-free dry products results in a certain amount of "denaturing."

The product obtained as just described and which we designate as Preparation I was used as the starting material for further purification. For the preparation of material referred to as Preparation II, it is convenient to dissolve in water the moist product after salting out with ammonium sulfate and then to proceed to precipitation with magnesium sulfate without removal of ammonium salts. After two or more precipitations with magnesium sulfate the precipitate is dissolved in water and the bulk of the magnesium sulfate removed by adding alcohol to 75 to 80 per cent concentration. The alcohol is then removed from the filtrate and the product precipitated once or twice more with ammonium sulfate until the precipitate contains no residual magnesium salts. It is then treated as previously described for the final steps of Preparation I. In its general properties preparations of Product II closely resemble those of Product I but, as already stated, on hydrolysis it is found that histidine has virtually disappeared and that the glutamic acid and glycine are diminished, though it is difficult to assert their absence with confidence. Product II gives a rather attractive Reineckate which is best prepared by adding its cold solution to a previously prepared cold solution of excess of Reinecke acid. It crystallizes in well formed prisms and is very sparingly soluble in water. After drying *in vacuo* over sulfuric acid, it yields very little (1.0 per cent) additional moisture on drying at 75°. The melting point, as might be anticipated, is not sharp. It begins to darken at about 200° and finally decomposes completely with evolution of gas between 240–245°. It gave the following results on analysis, dried at 65° *in vacuo*: chromium 5.88 to 5.95, carbon 39.7, hydrogen 5.54, sulfur 14.6, nitrogen 17.5 per cent.

The magnesium sulfate mother liquors from the preceding preparation were stirred with alcohol (80 per cent) to precipitate most of the salt and the alcoholic mother liquors concentrated and precipitated with Reinecke salt, which was then regenerated as before described. The general properties of this fraction (No. III) resembled those of Preparation II in most respects and its elementary composition was similar. The Reineckate had approximately the same composition and melting point: chromium 5.95, carbon 38.1, hydrogen 5.2, nitrogen 17.7 per cent. The preparation of Product IV by precipitation with flavianic acid requires little

comment. The amount of flavianic acid required for complete precipitation is a little over 50 per cent of the weight of the material of Product I. It is essential not to employ flavianic acid at an earlier stage of the process, as precipitation is then very incomplete. As already stated, the decomposition of the oily flavianate is difficult and requires the use of a considerable excess of sulfuric acid. The flavianic acid was extracted with amyl alcohol and ether and the residue worked up as usual.

The methods used for the identification of the amino acids resulting from the hydrolysis of our products do not for the most part offer anything new, and, since the separation of the products has been repeated many times, it will not be necessary to go into great detail. Hydrolysis was usually carried out by boiling with 20 per cent hydrochloric acid for at least 6 hours. A dark colored solution results in which black particles are usually observable. Every trace of glucosamine or other substance reducing Fehling's solution is destroyed in the process and frequently black particles will be found in the condenser tube, suggesting the formation of some volatile decomposition product. On evaporation of the solution under diminished pressure, taking up in water, and filtering a clear solution of the amino acids is readily obtained.

The hexone bases were separated first of all as phosphotungstates, and then, after regeneration and removal of ammonia, they were separated by the Kossel silver method, making use of the modification introduced by Vickery and Leavenworth.

*Histidine*--As has already been stated in the introduction, far more histidine is present in the crude material once precipitated by ammonium sulfate than in the more highly purified preparations which have been repeatedly salted out with magnesium sulfate. In the latter the amount of histidine estimated colorimetrically with the diazo reagent according to Koessler and Hanke (10) was not over 0.1 per cent, assuming that the whole of the color was due to histidine, an assumption that is probably incorrect. In these preparations it was not possible to obtain any crystalline derivative of histidine or obtain any evidence of its presence except that of a possible diazo reaction. On the other hand, in the case of the cruder preparations once salted out with ammonium sulfate an amount of histidine estimated colorimetrically at 2.5 to 4.0 per cent was observed, and it was easy on de-

composing the appropriate silver fraction to obtain histidine diflavianate in good crystalline condition and in amount equivalent to about 70 per cent of that indicated by the colorimetric tests. A typical example of the analysis for histidine of a sample of material once precipitated by ammonium sulfate may be cited. 7 gm. gave on hydrolysis and fractionation with phosphotungstic acid and silver, a histidine fraction containing 0.054 gm. of nitrogen, equivalent to 0.1992 gm. of histidine. On adding excess of flavianic acid (0.8 gm.), a yield of sulfur-yellow crystalline diflavianate, darkening at 235° and melting at 249–251°, equivalent to 0.14 gm. (*i.e.* 2.0 per cent on original material) of histidine was readily obtained. Nitrogen in dry salt, 12.4 per cent; calculated, 12.5 per cent.

*Arginine*—The arginine was estimated in the appropriate silver fraction by decomposing with hydrogen sulfide and then decomposing the arginine by prolonged boiling (6 hours) with 20 per cent sodium hydroxide. All of the analyses of the various preparations gave results between the limits of 11.5 and 15.0 per cent, with an average value of 13.2 per cent. The arginine was fully identified as picrolonate and as flavianate, the later crystallizing in glistening orange plates, decomposing without melting above 260°, and containing 16.9 per cent nitrogen (calculated, 17.2 per cent). An alternative method of estimating arginine without preliminary hydrolysis based on Sakaguchi's (11) method with edestin as standard gave 13.7 per cent arginine in Preparation II.

*Lysine*—The filtrates from the silver precipitates were worked up for lysine in the usual fashion. 10 gm. of Preparation II gave a filtrate containing nitrogen equivalent to 0.4584 gm. of lysine (4.6 per cent). On recovering the free base with phosphotungstic acid and treatment with alcoholic picric acid, a yield of crystalline lysine picrate equivalent to 0.35 gm. of lysine was obtained. On recrystallization from water the picrate separated in needles darkening above 230° and decomposing suddenly at 260–262°. Analysis: carbon 38.8, hydrogen 4.8; calculated, carbon 38.4, hydrogen 4.5 per cent.

*Leucine*—Although a considerable amount of leucine is present among the products of hydrolysis, it was not possible to isolate it directly in good yield and in pure condition. The following method is based on the isolation of leucine as its uramido acid, a

procedure which we have often employed before and found thoroughly satisfactory. 4 gm. (Preparation II) were hydrolyzed by boiling with 10 parts of 1:5 by volume sulfuric acid for 6 hours. The dark mixture was diluted 4-fold and precipitated with phosphotungstic acid. The precipitate was removed by filtration and the filtrate freed of both sulfuric acid and phosphotungstic acid by barium hydroxide. The filtrate was then concentrated to about 25 cc. Potassium cyanate (4 gm.) was then added by degrees while it was gently warmed on the water bath and the alkaline solution slowly concentrated to about half its bulk. It was then made just acid to Congo red with dilute hydrochloric acid and placed in the refrigerator overnight. The crystals, which weighed 0.40 gm. and melted at 202–204°, were filtered off. The filtrate was then extracted with ether for 24 hours in a continuous extractor. The extract on dissolving in water and concentrating to about 4 cc. gave an additional 0.22 gm. of crystals which melted around 190° rather indefinitely. The mother liquor on evaporation contained 0.38 gm. of solid material, of which about half could be recovered in crystalline form by evaporation with hydrochloric acid, so as to convert the uramido acid into hydantoin. The total yield of crude products from 4 gm. of starting material amounts to 0.62 gm. of uramido acid and 0.19 gm. of hydantoin, equivalent to 0.631 gm. of leucine or 15.8 per cent. Since under favorable conditions the yield of uramido acid from leucine is about 70 per cent, it would appear that the probable leucine content in our product is not far from 20 per cent.

The uramido acid after a single crystallization from water melted with effervescence at 205–206°. On analysis it gave the following results: carbon 48.8, hydrogen 7.8, nitrogen 15.8; calculated for  $C_7H_{14}O_3N_2$ , carbon 48.3, hydrogen 8.0, nitrogen 16.1 per cent. On evaporating the uramido acid with dilute hydrochloric acid, it was converted smoothly into isobutylhydantoin melting with slight sintering but without effervescence at 212°. This conversion is of importance, for isoleucine, if present, would give a hydantoin of much lower melting point. The hydantoin had a specific rotation of  $-86^\circ$  and on analysis gave satisfactory figures (carbon 53.4, hydrogen 7.8; calculated, carbon 53.2, hydrogen 7.70).

The uramido and hydantoin preparations just referred to gave only the barest trace of a faint yellow color on evaporation with

nitric acid and moistening the residue with ammonia. On the other hand, similar preparations from cruder material (Product I) gave a distinct orange color which we believe to be due to traces of phenylalanine. We have been unable to isolate any pure phenylalanine derivatives, but have obtained analyses indicative of a mixture of leucine and phenylalanine hydantoin.

Reference perhaps should be made to the fact that recently Boyd (12) on purely theoretical grounds has questioned the possibility of extracting the uramido acid of leucine with ether. We have made such frequent and successful use of the method that we are quite unwilling to accept this criticism as justified.

*Hydroxyproline*—The residue left after the preceding extraction of uramido acids chiefly derived from leucine was boiled for an hour with one-tenth its volume of concentrated hydrochloric acid in order to convert unextracted uramido acids into hydantoins. Continuous extraction with ether was then carried out for 36 hours. The ethereal solution was then allowed to stand for a couple of days in a cold place. A small amount of crystalline material separated out and was removed. This proved to be hydantoin acetic acid derived from aspartic acid. On recrystallization from water it separated in thick, highly refractive prisms melting at 220–223° with slight previous sintering. Analysis: carbon 38.2, hydrogen 3.90; calculated, carbon 38.0, hydrogen 3.80 per cent. No hydantoin propionic acid derivable from glutamic acid was encountered. On evaporation of the ether solution above referred to, a clear, syrupy residue weighing 0.78 gm. was obtained, which slowly set to a solid mass of thick shining prisms. The crystals were drained on porous tile and then recrystallized by dissolving in a minimum of alcohol and adding benzene. They melted at 162°. Four different preparations showed specific rotations of  $-98.0^\circ$ ,  $-96.8^\circ$ ,  $-93.5^\circ$ , and  $-97.0^\circ$ . These figures compare with a melting point of 162–165° and a specific rotation of  $-97.2^\circ$  for the hydantoin of hydroxyproline prepared from gelatin. Analysis: nitrogen, 18.1; calculated, 18.0 per cent. A simpler and more direct method of separating hydroxyproline was later adopted, based on the removal of arginine with flavianic acid, followed by precipitation with Reinecke acid. On decomposition of the Reineckate with dimethylaniline a yield of hydroxyproline, based on polarimetric estimation, of slightly over 10 per cent was obtained. It was



further identified by its naphthalenesulfonic acid derivative, m.p. 88°.

*Glycine*—Glycine was easily identified among the products of hydrolysis of the crude material precipitated by Reinecke acid before salting out with ammonium sulfate. In the preparations further purified by salting out, it was difficult to separate glycine as the ester hydrochloride, although we have obtained the characteristic naphthalenesulfonic acid derivative. Owing to the kindness of Dr. Max Bergmann we have been able to make use of an unpublished method for the determination of glycine and have assured ourselves that Preparation II does not contain more than 4 to 6 per cent.

The glycine from the crude preparations was isolated in the usual way as glycine ethyl ester hydrochloride, which melted at 144° and contained 10.5 per cent nitrogen (calculated, 10.1). The free amino acid melted with discoloration at 230° and contained 18.2 per cent nitrogen (calculated, 18.6). It was also identified as the naphthalenesulfonic acid derivative melting at 153–155°.

*Dicarboxylic Acids*—These acids were separated as calcium salts after hydrolysis with hydrochloric acid. After purification with phosphotungstic acid and silver sulfate according to Foreman's method (13), it was found that as much as 25 to 30 per cent of the total nitrogen was in the dicarboxylic acid fraction, all of the nitrogen being in the  $\text{NH}_2$  form. This estimate is undoubtedly too high, owing to the simultaneous precipitation of some monamino acids. The acids derived from crude preparations, either before salting out or after a single salting out with ammonium sulfate, readily gave crystals of *d*-glutamic acid hydrochloride on saturation with hydrogen chloride. Melting point, 198–200°; nitrogen 7.4 to 7.6 per cent; calculated, nitrogen 7.63 per cent. From similar material that had been partially racemized by cold sodium hydroxide the optically inactive hydrochloride melting at 203° was isolated in good quantity. But as already stated, on further purification of the original material either by magnesium sulfate or flavianic acid, it was found that crystalline glutamic acid hydrochloride could no longer be separated. On boiling the mixed acids with excess of zinc oxide and assuming, unjustifiably, that the whole of the nitrogen in the sparingly soluble zinc salt was glutamic acid, we obtained maximal values of 1.25 per cent glu-

tamic acid in Preparations II and IV. On the other hand, these preparations gave good yields of aspartic acid (17 per cent and over) separated as basic lead salt, as copper salt, and as free acid. Analysis: carbon 36.6, hydrogen 5.40, nitrogen 10.4; calculated, carbon 36.8, hydrogen 5.26, nitrogen 10.5 per cent. Aspartic acid was also identified as hydantoin acetic acid, m.p. 220–223° (*cf.* section on hydroxyproline). Most of the aspartic acid was racemized, presumably during hydrolysis. After the removal of glutamic and aspartic acids as zinc and lead salts respectively, indications of a dicarboxylic acid easily soluble in water and giving a very soluble copper salt precipitable by alcohol were obtained. The identification is still incomplete.

*Aminohexose*—It has already been stated earlier that so far we have been entirely unable to isolate glucosamine hydrochloride as such. We have confirmed Neuberg and Kerb's statements as to the precipitation of glucosamine with mercuric acetate and soda and have tried, unsuccessfully, to make use of this fact in isolating glucosamine from partly hydrolyzed products which had been previously treated with phosphotungstic acid and also neutral mercuric acetate.

Furthermore, we have tried the hydrolysis of chloroform-soluble products obtained from the acetolysis of our substance, but apparently basic substances still remain in combination with the aminohexose and vigorous hydrolysis destroys the latter. As already stated we have made use of Elson and Morgan's method for making a very tentative estimation of glucosamine. The colorimetric tests were made on solutions that had been heated for 45 minutes or longer in a steam bath with *N* hydrochloric acid. It must be admitted that this extension of Elson and Morgan's method may prove to be beset with serious errors, but at the moment it seems the most useful procedure available. The colors obtained with our products are not nearly as stable as those obtained with pure glucosamine and should be matched within 10 minutes.

Reference has also been made to the isolation of phenylglucosazone from our products on heating with phenylhydrazine in 5 per cent acetic acid. The material may be previously partially hydrolyzed with *N* hydrochloric acid but this is not essential. No separation of osazone takes place until after about 20 minutes and

thereafter the separation steadily increases. Alanylglucosamine anhydride and also acetylglucosamine behave similarly, while glycyglucosamine gives nothing but a little oily separation. The phenylglucosazone, when filtered off and washed, melts above 200° in the crude state and after recrystallization from alcohol at 205–206°. Analysis: carbon 60.8, hydrogen 6.15, nitrogen 15.6; calculated, carbon 60.3, hydrogen 6.14, nitrogen 15.6 per cent. The identity of the phenylglucosazone was further established by its correct levorotation in pyridine and alcohol mixture. It may be noted that *p*-nitrophenylhydrazine, which apparently does not easily yield an osazone with glucosamine, under similar conditions gave no trace of osazone when heated with solutions of our products, either before or after hydrolysis.

*Peptic Digestion*—Substrate, 0.25 gm., of Preparation II, containing 37.5 mg. of nitrogen. The ferment solution contained 0.25 gm. of active pepsin in 5 cc. of 0.1 N hydrochloric acid. Toluene was used as an antiseptic. Temperature, 35°. The pepsin solution alone showed no increase in amino nitrogen on digestion.

	Increase in NH <sub>2</sub> -N (Van Slyke)
hrs.	mg.
20	1.02
48	1.80
96	2.40
148	3.24

A second experiment was made with crystalline pepsin for which we are indebted to Dr. R. M. Herriott. Substrate, 0.466 gm., of Preparation II, containing 70 mg. of nitrogen. Temperature, 35°.

	Increase in NH <sub>2</sub> -N (Van Slyke)	
	Blank	Substrate
	mg.	mg.
30 hrs.	1.10	3.66
2 days	1.33	5.04
4 "	2.46	7.44
6 "	2.66	7.98
14 "	3.10	9.18

In both the above experiments the increase on prolonged digestion with pepsin resulted in an increase of amino nitrogen equivalent to only 8.6 and 8.7 per cent of the total nitrogen. Such a small change over so long a period with a large excess of ferment leads to the conclusion that the action of pepsin is slight and slow. The change, however, seems to be a definite one, for incubation with 0.1 N acid alone and no enzyme gave absolutely no change in the amino nitrogen. No free glucosamine appears to be liberated in the course of digestion. The survival of some clinical activity after peptic digestion has already been mentioned (Case 433695).

*Erepsin Digestion*—Glycerol extract of fresh pig intestinal mucosa (10 cc.). Substrate, 0.45 gm., of Preparation II, containing 67.5 mg. of nitrogen. Total volume, 50 cc. Temperature, 35°. Toluene was used as a preservative.

	Increase in $\text{NH}_2\text{-N}$ (Van Slyke)	
	Blank	Substrate
	mg.	mg.
4 hrs.		6.6
1 day	3.9	9.9
4 days	8.1	26.7
10 "	8.4	36.0
20 "	9.0	43.2

Allowing for the increase in the blank experiment, about 51 per cent of the total nitrogen of the substrate was in the amino form at the end of 20 days. In another similar experiment 58 per cent increase was observed. The digestion is slow but tolerably complete.

In conclusion, one of us (H. D. D.) wishes to record his grateful appreciation of the opportunity accorded him to cooperate in the present work by Dr. W. W. Palmer. While Dr. Palmer is in no way responsible for any of the results recorded in this paper, it is equally true that if it were not for his constant encouragement the work would have been long since abandoned owing to the many difficulties and disappointments that were encountered. We are also indebted to Dr. Hans T. Clarke for generous cooperation and for allowing us the facilities of his department, including the execu-

tion of many microanalyses by Mr. William Saschek. To Dr. Max Bergmann we owe our thanks for a specimen of glycylglucosamine hydrochloride and also for generously permitting us to use a method of glycine estimation which he has not yet published.

#### SUMMARY

The preparation from commercial liver extract of products clinically active in causing remission in pernicious anemia is described. The method is based on the removal of much relatively inactive material by precipitation with alcoholic calcium acetate, followed by precipitation of the active material with Reinecke acid. The decomposition of the Reineckate requires special methods which are described. Subsequent purification is effected by salting out the active material with ammonium sulfate and later by the use of either magnesium sulfate, sodium chloride, or of flavianic acid. 30 mg. of the product caused a perceptible reticulocyte response in suitable pernicious anemia patients, while 80 mg. have given a maximal response.

Under suitable conditions substantially the whole of the active material is precipitable by ammonium sulfate since none could be recovered from the filtrate. Precipitation in the magnesium sulfate is quantitatively less complete. The yield of purified product approximates 1 per cent of the dry liver extract.

The clinical activity of the product is readily abolished by exposure to cold 0.5 N sodium hydroxide and by boiling for 1 hour with 0.5 N sulfuric acid and also by salts of heavy metals. Exposure to alkali involves extensive racemization.

On hydrolysis of the active material the following products were obtained. (a) An aminohexose similar to glucosamine but not definitely identified as such. It gave phenylglucosazone on treatment with phenylhydrazine and pyrrole derivatives on condensation with acetylacetone and ammonia on treatment with alkali. Chondrosamine was absent. (b) The following amino acids were present in all preparations so far obtained: lysine, arginine, glycine, leucine, hydroxyproline, aspartic acid. Crude preparations contained in addition, histidine, glutamic acid, and possibly traces of phenylalanine. No other carbohydrate or amino acid groupings were detected. The product unlike glucosamine gives a positive Molisch reaction, but we find that glucosamine peptides behave

similarly. Pyrimidine or purine bases were absent. Reference is made to the possible molecular weight of the product, to its decomposition by pepsin and by erepsin, and to its relationship with previously described products. No claim to strict chemical individuality is advanced, since the separation of aminohexose polypeptides must of necessity be extremely difficult.

### *Clinical Tests*

The following clinical tests (see the protocols at the end of this paper) are arranged in the order in which they are referred to in the text. With one exception all of the cases referred to were in the Medical Clinic, Presbyterian Hospital. In order to economize space, clinical histories are not included, but the case number is given in each case so that the details are on permanent record. It will be noted that in a number of instances in which the material undergoing examination was found to be inactive, the suitability of the case from the clinical point of view was confirmed by subsequent injection of known potent material, resulting in a normal reticulocyte response.

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Day	Case 392148		Case 364172		Case 415857		Case 417195	
	Red blood cells <i>millions</i>	Reticulocytes <i>per cent</i>	Red blood cells <i>millions</i>	Reticulocytes <i>per cent</i>	Red blood cells <i>millions</i>	Reticulocytes <i>per cent</i>	Red blood cells <i>millions</i>	Reticulocytes <i>per cent</i>
1	1.3*	1.3	2.0*	2.8	1.2*	2.6	1.4*	6.2
2		1.4		3.0		3.2		3.3
3				5.0		3.1		2.6
4					†	1.7		2.9
5		0.8		24.2		4.2	†	3.4
6	†	2.6		40.8		21.5	1.5	3.4
7		1.8		31.6		33.0		3.7
8		1.7	2.6	27.4	1.6	41.0		11.0
9		6.3				72.4		26.8
10		14.8				51.4		33.1
11		20.2					1.7	31.2
12	1.1	11.3				32.9		29.1
13		8.0			2.4	31.0		9.9
14	1.3					11.0		
	* Filtrate from pptn., 20 cc. Lilly's parenteral liver extract with Reinecke acid		* 150 mg. subcutaneously, product from decomposition of crude Reineckate ppt. +		* 100 mg. intravenously, Reineckate ppt. from alcoholic CaCl <sub>2</sub> ppt. +		* 80 mg. intravenously, ammonium sulfate filtrate + 80 mg. intravenously, ammonium sulfate ppt. Filtrate -; ppt. +	
	† Reineckate ppt. Filtrate -; ppt. +				Reineckate from filtrate from alcoholic CaCl <sub>2</sub> pptn. Ca ppt. -; Ca filtrate +			

Day	Case 356184		Case 316263		Case 419573		Case 423439	
	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes
	millions	per cent	millions	per cent	millions	per cent	millions	per cent
1	1.1	3.6	1.5*	3.6	1.6*	11.1	*	5.5
2	*	3.6		5.4		8.3	1.5	3.9
3		4.9		6.8		9.8		6.0
4		6.2		6.7		17.3		8.5
5		8.0		5.5		28.0		27.7
6		11.8		6.9	2.6	24.0	1.8	19.3
7		34.7	1.7†	5.7				52.6
8	1.6	29.0		8.2				42.1
9		25.8		5.5				28.4
10				6.4			2.8	28.3
11				3.2				
11				4.4				
13				3.4				
	* 105 mg. intravenously; repeated ammonium sulfate pptn. +		* 60 mg. intravenously, ammonium sulfate ppt. † 60 mg. intravenously, ammonium sulfate filtrate Ppt. doubtful slight +; filtrate — On subsequent treatment reticulocytes rose to 45%		* 100 mg. subcutaneously; repeated ammonium sulfate pptn. +		* 80 mg. subcutaneously; thrice repeated ammonium sulfate pptn. +	



Day	Case B. H.		Case 76032		Case 297466		Case 81298	
	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes
	millions	per cent	millions	per cent	millions	per cent	millions	per cent
1	1.4	1.3	1.4*	5.0			2.6*	2.5
2	*	1.1			1.4*	4.3		1.9
3		1.9		1.9		2.1		2.5
4		2.6		2.6		3.1		9.5
5		8.0		11.3		3.6		
6	2.2	13.2		13.1		23.4	2.6	12.7
7		25.0		21.9		31.3		9.3
8		22.0		10.0	2.3	25.2		8.3
9			2.1	14.7		27.2		7.8
10				8.5		36.9		5.4
11					2.7	13.2		
	* 150 mg. intravenously, picric acid ppt. +		* 80 mg. subcutaneously, flavianic acid ppt. Positive but response not maximal as shown by later 41.7% reticulocytes		* 80 mg. intravenously, MgSO <sub>4</sub> ppt. +		* 60 mg. intravenously, MgSO <sub>4</sub> ppt. +	

Day	Case 71632		Case 237059		Case 429486		Case 382735	
	Red blood cells millions	Reticulocytes per cent	Red blood cells millions	Reticulocytes per cent	Red blood cells millions	Reticulocytes per cent	Red blood cells millions	Reticulocytes per cent
1	0.9*	3.0	1.9*	3.3	3.2*	3.4	2.5*	2.4
2		2.5				3.7		
3	0.6	5.7				3.4		
4		5.5		4.7		6.4		
5	1.2†	4.3		7.2		6.5		5.9
6		2.6	1.7†	6.7		8.0		4.3
7		5.4			4.0	5.8	2.2†	4.5
8		28.8						5.1
9		49.2						4.1
10		69.7		15.2				7.6
11		35.6		6.2				6.0
12	2.1	28.0		8.5				12.9
13								8.6
14								17.3
15							2.8	16.5
	* 50 mg. subcutaneously, MgSO <sub>4</sub> ppt.		* 30 mg. intravenously, MgSO <sub>4</sub> ppt.		* 120 mg. intravenously, MgSO <sub>4</sub> filtrate		* 50 mg. subcutaneously, MgSO <sub>4</sub> filtrate	
	† Transfusion and 40 cc. parenteral liver extract		† 90 mg. intravenously of same		Slight +		† 120 mg. intravenously, MgSO <sub>4</sub> ppt.	
			Moderate +				Ppt. +	

Day	Case 442154		Case 433695		Case 354091		Case 389711	
	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes	Red blood cells	Reticulocytes
	millions	per cent	millions	per cent	millions	per cent	millions	per cent
1	1.6*	1.3	2.3*	2.5	1.1*	3.3	1.8*	4.3
2		1.2		1.9		2.4		
3				4.0		1.8		
4		1.9		4.6		1.1		1.9
5		8.1		6.8	0.8†	0.9		
6		19.1		<b>14.8</b>		0.4	1.7†	1.0
7		20.0	2.3†	7.7		0.2		
8		<b>23.9</b>		4.5	†	6.9		
9	2.2	19.0		6.5		29.8		
10				<b>9.8</b>		48.7		17.6
11	†	11.3		9.0	†	55.4		
12				7.1		<b>56.1</b>		
13						46.2	2.2	<b>29.9</b>
14								
15								
	* 115 mg. subcutaneously, neutral NaCl ppt. † Parenteral liver extract (10 cc.) gave no further response +		* 225 mg. subcutaneously, peptic digest of MgSO <sub>4</sub> ppt. † 20 cc. Lilly's parenteral extract +		* 150 mg. subcutaneously, Reineckate from autolyzed liver extract † 10 cc. parenteral liver extract Autolyzed product, -		* 120 mg. intravenously, Ba salt from Reineckate † 110 mg. active Reineckate Ba salt, -	

# THE APPLICATION TO THE COLORIMETER OF THE SCHOENHEIMER AND SPERRY METHOD FOR THE DETERMINATION OF TOTAL AND FREE CHOLESTEROL\*

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In 1934 Schoenheimer and Sperry (1) presented a micromethod for the determination of free and combined cholesterol in blood and other biological material. Using a Zeiss Pulfrich photometer to measure the color developed by the Liebermann-Burchard reaction with small amounts of cholesterol digitonide, these authors claim a degree of accuracy previously obtainable only with the macrogravimetric procedure of Windaus.

In principle, the method consists in the precipitation of cholesterol from an acetone-absolute alcohol extract with digitonin. The digitonide is freed of other chromogenic substances and then subjected to a color reaction. For details of the procedure the reader is referred to the communication of Schoenheimer and Sperry.

In adapting the method to the use of a laboratory not having access to a sensitive photometer, certain obstacles were encountered. With an ordinary colorimeter equipped with microcells and a suitable color filter,<sup>1</sup> it was found that the mean difference between duplicate determinations in blood sera was often in excess of 10 per cent. Examination of preliminary analyses revealed at least two difficulties. First, the inability to match satisfactorily with a suitable standard the very faint colors developed with samples containing less than 0.05 mg. of cholesterol. This was

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<sup>1</sup> A Wratten color filter, No. 71-a (mounted by the Klett Manufacturing Company, New York), was placed over the eyepiece of the colorimeter.

especially true of the free cholesterol fraction. The second difficulty had to do with the preparation of a standard which would permit the reading of several samples. Schoenheimer and Sperry state that with the use of a suitably equipped colorimeter, the standard deviation of percentage errors of thirty-three determinations was 5 per cent and in one case (0.025 mg. of cholesterol) was 12 per cent.

To overcome the first of the difficulties mentioned above, experiments were planned to determine the least amount of cholesterol that could be read in the colorimeter with a standard deviation not exceeding 3 per cent. Series of determinations of known

TABLE I

*Degree of Variation Observed in Determining Graduated Known Amounts of Cholesterol in the Colorimeter*

Amount of cholesterol in sample*	Limits of variation	Standard deviation	No. of determinations
mg.	mg.	per cent	
0.020	0.018-0.022	5.27	14
0.040	0.037-0.043	4.68	14
0.060	0.058-0.063	2.46	16
0.080	0.079-0.082	0.91	15
0.100	0.098-0.103	1.24	22

\* These samples were prepared by dissolving weighed amounts of anhydrous cholesterol in acetic acid (Eastman Kodak Company).

amounts of cholesterol varying from 0.02 to 0.10 mg. were carried out. The results obtained are shown in Table I.

From the data in Table I it is evident that samples of serum extracts should contain in excess of 0.05 mg. of cholesterol in order to be measured satisfactorily in the colorimeter with a precision of less than 3 per cent standard deviation. To obviate this difficulty the original extract is prepared by using 0.5 cc. of serum made up to 10 cc. with acetone-absolute alcohol solution. In addition, a 3 cc. portion of the filtrate is used for the free cholesterol fraction, whereas the original method advocates the use of 2 cc. in this part of the procedure. With this modification, the average total cholesterol aliquot contains in excess of 0.08 mg.,

and the free cholesterol aliquot more than 0.07 mg. of cholesterol. A standard containing 10 mg. of cholesterol per 100 ml. of acetic acid is employed. In the event that the total serum cholesterol can be anticipated to be less than 125 mg. per 100 ml., more serum is used in making the original extract.

Upon the addition of measured amounts of acetic anhydride and sulfuric acid to cholesterol solutions, the color developed is affected by several factors, the most important of which are temperature, time, and intensity of light (2-5). Mirsky and Bruger (6) believed temperature control to be the most important single

TABLE II

*Degree of Fading of Standard Solutions in Relation to Time When Exposed to the Light and Heat of a Colorimeter*

0.10 mg. of cholesterol was used throughout.

Time	Amount determined (mean)	Deviation	No. of determinations
min.	per cent	per cent	
27	100.4	+0.4	14
29	99.8	-0.2	14
31	99.0	-1.0	14
33	94.7	-5.3	14
35	92.4	-7.6	11
37	92.1	-7.9	8

Exposures of approximately 50 seconds each were made at 2 minute intervals.

factor in the Liebermann-Burchard color reaction for cholesterol. Schoenheimer and Sperry found with their procedure that when 0.1 cc. of  $H_2SO_4$  was added, the temperature being kept constant at  $25^\circ$  and the light excluded, the maximum color was reached at about 27 minutes and fading started at about 37 minutes. When higher temperatures were used, fading started earlier. These authors suggest that when a colorimeter is used, it may be necessary to employ more than one standard during a series of readings; however, no data were presented to indicate the stability of standard solutions when used in the colorimeter.

The solution in the standard cell of the colorimeter is subjected to repeated exposures of intense light and of constant heat by

radiation and convection; with the photometer these factors, with the single exception of heat by radiation, are eliminated. In our hands it was found that standard solutions began to fade appreciably after 4 minutes when in the standard cell, thereby permitting at most three determinations. It may be seen in Table II that the loss of color becomes more pronounced with further light and heat exposures.

A standard containing 5 times the volume of a single standard has been employed in order to obviate this fading effect, to offset the variation that occurs between duplicate standards, and to

TABLE III

*Duplicate and Triplicate Results Obtained with Blood Sera with the Schoenheimer and Sperry Procedure Modified for the Colorimeter*

Sample No.	Total cholesterol		Maximum deviation from mean	Free cholesterol		Maximum deviation from mean
	In sample	Per 100 cc. serum		In sample	Per 100 cc. serum	
	mg.	mg.	per cent	mg.	mg.	per cent
I	0.0752	150.4	0.33	0.0678	45.2	0.51
	0.0757	151.4		0.0685	45.7	
II	0.0862	172.4	0.70	0.0840	56.0	0.67
	0.0855	171.0		0.0847	56.5	
	0.0851	170.2		0.0837	55.8	
III	0.1105	221.0	0.27	0.1064	70.9	0.23
	0.1111	222.2		0.1069	71.3	
IV	0.1342	268.4	0.34	0.1307	87.1	0.90
	0.1333	266.6		0.1282	85.5	
V	0.1639	327.8	0.81	0.1460	97.3	1.64
	0.1613	322.6		0.1492	99.5	
	0.1626	325.2		0.1449	96.6	

save time and materials. This large standard is prepared and placed in the water bath with the unknown samples. For each determination in a series of unknowns, a fresh portion is transferred to the standard cell of the colorimeter. It has not been found necessary to wash out the standard cell after each filling. By this procedure the standard remains constant for the 27 to 37 minute interval, giving ample time to make six to eight determinations of five readings each. Typical results obtained with blood sera by this modified procedure are shown in Table III.

## SUMMARY

An adaptation for the colorimeter has been made of the Schoenheimer and Sperry method for cholesterol determination. In the preparation of the serum extract 0.5 cc. of blood serum is made up to 10 cc. with the acetone-absolute alcohol solution, whereas the original procedure advocates the use of 0.2 cc. made up to 5 cc. By so doing, 1 cc. portions of the filtrate generally contain more than 0.05 mg. of total cholesterol. For the free cholesterol fraction, 3 cc. of the extract and 1.5 cc. of the digitonin solution are used in place of 2 cc. of the extract and 1 cc. of digitonin solution as called for in the original method. The precipitation of the cholesterol, the washing of the digitonide, and the development of the color are carried out as presented in the Schoenheimer and Sperry communication. In place of using a new standard for each or for every few samples, a standard containing 5 times the volume of a single standard is employed, and fresh portions are transferred to the standard cell for each determination. With these minor modifications for use with the colorimeter, duplicate and triplicate determinations have been obtained with maximum deviations from the mean of less than 3 per cent.

Drs. Schoenheimer and Sperry extended every courtesy and made valuable suggestions throughout this study. Mr. Oscar Keller rendered valuable technical assistance, for which grateful recognition is given.

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# STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

## I. THE SYNTHESIS OF CERTAIN TETRAVALENT AMINO ACIDS AND THEIR DERIVATIVES

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In the study of the physicochemical properties of the proteins, it is often convenient to consider the behavior of the simpler ampholytes which reflect to some degree the properties of the more complex protein molecules. The investigation of simpler synthetic amphoteric models, usually amino acids and peptides, has illuminated such diverse properties of proteins as electrolytic dissociation (9, 13), apparent molal volume (7), solubility (6), and behavior toward enzymes (1).

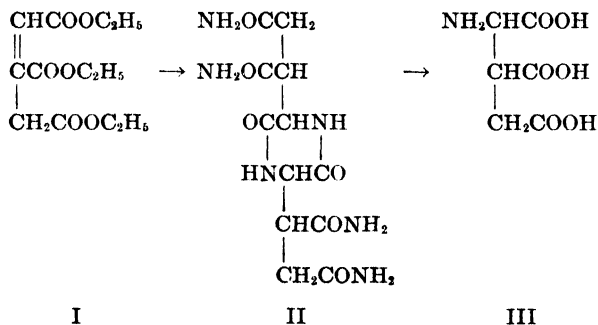
From the researches of Herricott and Northrop on pepsin (14) and of Freudenberg and Eyer (12), Jensen (15), and du Vigneaud (21) on insulin, it seems likely that the apparently specific properties of many proteins may inhere in certain groups or linkages in the molecule. Many of these groups or linkages may be reproduced in synthetic models. Whether these groups may completely function dissociated from the colloid moiety itself cannot as yet be answered. It would seem of interest, however, to prepare a series of complex synthetic ampholytes and their derivatives and to examine several of their properties. These substances will resemble the protein in bearing several free amino, carboxyl, and other characteristic groups upon a relatively small molecular area. The present communication is concerned with the synthesis of three new tetravalent amino acids:  $\alpha, \gamma, \delta$ -triamino- $\Delta^{7,8}$ -pentic acid,  $\alpha$ -aminotricarballylic acid, and  $\epsilon, \epsilon'$ -diamino-di( $\alpha$ -thio-*n*-caproic acid). The latter substance was further characterized by the formation of its  $\epsilon, \epsilon'$ -diguanido and  $\epsilon, \epsilon'$ -diphenylureido

derivatives. The glycylpeptide of  $\alpha$ -aminotricarballylic acid was also prepared. Triamino acids possessing three amino groups and one carboxyl group, such as the dipeptides lysyllysine and  $\alpha,\beta$ -diaminopropionic acid dipeptide, have been synthesized by Fischer and Suzuki (11). The peptide glutamylglutamic acid, containing one amino group and three carboxyl groups and hence analogous to the above  $\alpha$ -aminotricarballylic acid or its peptide, has been prepared by Bergmann and Zervas (2). The triamino acid and the tricarboxylic acid, whose syntheses are described below, were chosen for a proposed later synthesis of a hexapole dipeptide.

The synthesis of the dipeptide tetrapole, lysylglutamic acid, by Bergmann, Zervas, and Greenstein (3) has yielded a compound fairly soluble in cold water and a few of its electrochemical properties have been described by the author (13). In the endeavor to prepare a tetrapole which in addition to the property of being highly soluble in water would also contain the characteristic disulfide linkage of cystine, the synthesis of  $\epsilon,\epsilon'$ -diamino-di( $\alpha$ -thio-*n*-caproic acid) was undertaken. The wide spacing of the charged groups in the latter compound results in its highly polar character and consequent aqueous solubility.

Certain physicochemical and physiological properties of these substances and their derivatives will be described in later communications.

*$\alpha$ -Aminotricarballylic Acid*



When  $\beta$ -hydroxy acids are treated with a dehydrating agent, they pass over into  $\alpha,\beta$  unsaturated acids. On treatment of the

latter with ammonia, the corresponding amino acid should result, as was first indicated by Engel (10). The yields, however, were quite poor and later Morsch (18) and Dunn and Fox (8) employed for the syntheses of  $\beta$ -aminobutyric acid and of aspartic acid respectively not the unsaturated acid, but its corresponding ester. The latter method was followed in the synthesis of  $\alpha$ -aminotricarballylic acid, starting with aconitic acid triethyl ester (I) derived from citric acid.

#### EXPERIMENTAL

*Anhydroaminotricarballylic Acid Tetraamide (II)*—Samples of 16 gm. of triethyl aconitate (19) were placed in each of six 200 cc. pressure flasks and 150 cc. of absolute alcohol saturated with dry  $\text{NH}_3$  gas at  $0^\circ$  were added to each flask. The flasks were then kept at  $100^\circ$  for 6 hours and allowed to cool slowly to room temperature. The anhydro- $\alpha$ -aminotricarballylic acid tetraamide crystallized out on the sides of the flasks in yellowish clumps. This was filtered off, washed with cold water several times, then with alcohol, and finally dried *in vacuo*. The yield of crude substance, melting at  $217^\circ$ , was 19 gm. The alcoholic mother liquor yielded on evaporation approximately 11 gm. of impure tetraamide. For analysis a small amount was dissolved in lukewarm water, shaken with norit, and filtered. To the filtrate was added absolute alcohol to turbidity. On cooling in the ice chest, long colorless needles separate. M. p.,  $232^\circ$  with decomposition.

$\text{C}_{12}\text{H}_{18}\text{N}_6\text{O}_6$  (342.14)

Calculated. C 42.08, H 5.30, N 24.55, amide N as  $\text{NH}_3$  19.8

Found. " 42.22, " 5.40, " 24.48, " " " " 19.2

*$\alpha$ -Aminotricarballylic Acid (III)*—175 gm. of the combined crude anhydroaminotricarballylic acid tetraamide were dissolved in 600 cc. of 5 N NaOH and boiled under a reflux in an oil bath for 4 hours. At the end of this time the solution was chilled, diluted, and neutralized to methyl red with 10 N HCl. At this point a precipitate amounting to a few hundred mg. appeared. This was filtered off and not further investigated. To the filtrate, decolorized with norit, was added a hot solution of 250 gm. of copper acetate in 2500 cc. of water. A voluminous precipitate of the blue Cu salt immediately appeared, which was filtered off and

washed with water. Inasmuch as this salt was extremely insoluble in all solvents, it was thoroughly dried and ground up to a powder, and shaken on the machine with large volumes of water until the decanted wash water yielded a negative test for chloride ion. The yield of dried copper salt amounted to 90.0 gm. It was suspended in water and decomposed with  $\text{H}_2\text{S}$  and the clear colorless filtrate achieved by the use of norit was evaporated *in vacuo*. In concentrated solution the separation of the amino acid began. It was removed to a filter and washed several times with water. The yield of crude  $\alpha$ -aminotricarballylic acid, m.p.  $185^\circ$ , was 30 gm. The acid was dissolved in hot water and hot alcohol added slowly to turbidity. On cooling there crystallize beautiful flat gleaming plates. The yield of the pure substance melting at  $196^\circ$ , with decomposition, was 24.5 gm. This is around 12 per cent of the theory, based on the aconitic acid triethyl ester.

The amino acid is slightly soluble in cold water, easily in warm water, acids, and alkalies, and insoluble in organic solvents. In water solution it turns Congo red paper blue.

$\text{C}_6\text{H}_5\text{NO}_6$ (191.07).	Calculated.	C 37.68,	H 4.75,	N 7.32,	$\text{NH}_2\text{-N}$ 7.32
	Found.	" 37.95,	" 5.19,	" 7.11,	" 7.58

*Carbobenzoxylglycyl- $\alpha$ -Aminotricarballylic Acid*—A sample of 9.3 gm. of carbobenzoxylglycine (2) was suspended in 70 cc. of dry ether and treated with 10 gm. of fresh  $\text{PCl}_5$ . The mixture was shaken at  $0^\circ$  for about 15 minutes and then filtered. The filtrate was evaporated *in vacuo* to a syrup, the latter shaken out several times with dry cold petroleum ether to remove the oxychloride, and then taken up in 40 cc. of dry ether and immediately employed for the synthesis.

An amount of 7.3 gm. of  $\alpha$ -aminotricarballylic acid was dissolved in 60 cc. of 2 N NaOH and chilled in an ice bath. With vigorous shaking, the above ether solution of carbobenzoxylglycyl chloride was added alternately with 40 cc. of 2 N NaOH to the alkaline solution of the amino acid. When the reaction after 20 minutes was at an end, 5 N HCl was added to Congo blue, the ether layer removed, and the aqueous layer shaken out several times with ethyl acetate. The ethyl acetate extracts were combined, washed well with water, dried, and evaporated *in vacuo* at a low temperature to dryness. The residue was a thick colorless syrup. After

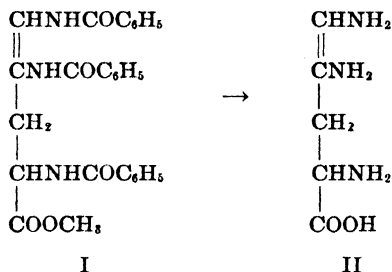
thorough drying in a vacuum desiccator, it set to a white solid. The yield amounted to 7.0 gm.; m.p., 72° with decomposition.

$C_{18}H_{18}O_9N_2$  (382.14). Calculated, N 7.33; found, N 7.20

*Glycyl- $\alpha$ -Aminotricarballylic Acid*—A sample of 6.5 gm. of carbobenzyglycyl- $\alpha$ -aminotricarballylic acid in 20 cc. of methyl alcohol and 10 cc. of water plus a few drops of acetic acid was catalytically hydrogenated in the presence of palladium. After about 20 minutes the reaction was at an end and the catalyst plus the crystallized peptide was filtered off. The material was separated from the palladium by washing with warm water and the combined filtrates evaporated *in vacuo* to dryness. The residue was taken up in the minimum amount of warm water, filtered, and treated with an equal volume of absolute alcohol. An oil separated which after standing at 0° for several hours crystallized into beautiful sheaves of prisms. The yield, amounting to 4.1 gm., was practically quantitative; m.p., 195° with foaming. The peptide is insoluble in cold water and in organic solvents, but soluble in hot water. Its aqueous solution is acid to Congo red paper.

$C_8H_{12}O_7N_2$  (248.1). Calculated. C 38.64, H 4.88, N 11.28  
Found. " 38.31, " 5.04, " 11.35

$\alpha, \gamma, \delta$ -Triamino- $\Delta^{\gamma, \delta}$ -Pentenic Acid



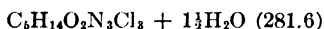
It was found by Kossel and Edlbacher (17) that treatment of histidine ester with an excess of benzoyl chloride resulted in an opening of the imidazole ring with the formation of a tribenzoyl unsaturated aliphatic ester (I). These authors did not go beyond the formation of this derivative of histidine. It was found that

simple splitting off of the benzoyl groups from the  $\alpha, \gamma, \delta$ -tribenzoyltriamino- $\Delta^{\gamma, \delta}$ -pentic acid methyl ester (I) by means of concentrated hydrochloric acid resulted in the formation of the corresponding free triamino acid (II) as the trihydrochloride salt.

To 9.4 gm. of  $\alpha, \gamma, \delta$ -tribenzoyltriamino- $\Delta^{\gamma, \delta}$ -pentic acid methyl ester was added a mixture of 150 cc. of concentrated HCl and 20 cc. of water and the entire mass was boiled for 5 hours under a reflux condenser. The solution is chilled, filtered from the benzoic acid, and the filtrate shaken out twice with ether.

The aqueous acid solution yields a negative reaction with diazotized sulfanilic acid. It was taken down to dryness *in vacuo* and the dark colored residue taken up several times with water and evaporated each time to dryness. Finally the solution was taken to dryness in a vacuum desiccator over  $P_2O_5$  and solid NaOH. The residue was a black, gummy mass. On treatment with acetone it solidified and was ground up to a fine powder in a mortar with absolute alcohol. The black color is completely extracted by the alcohol, leaving the substance a dirty gray. This was filtered off and washed several times with alcohol. The yield of the crude material was 2.2 gm., or 60 per cent of the theory. The substance is dissolved in an excess of hot methyl alcohol and filtered. To the filtrate dry ether was added drop by drop until precipitation was complete. The triamino acid separated as the trihydrochloride salt in large rhomboid prisms. The melting point was 171–173°, the substance turning brown and foaming.

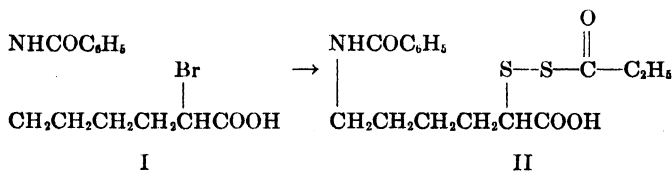
The substance decolorizes bromine water. It yielded a copious precipitate with phosphotungstic acid.



Calculated. C 21.30, H 6.06, N 14.92, Cl 37.7

Found. " 21.16, " 6.48, " 15.16, " 37.2

$\epsilon, \epsilon'$ -Diamino-Di( $\alpha$ -Thio-*n*-Caproic Acid)





III



IV



V

The starting material for the synthesis of this substance was  $\epsilon$ -benzoylamino- $\alpha$ -bromo-*n*-caproic acid (I) (5). Several methods are available for the replacement of the  $\alpha$ -halogen by sulfur and after several trials the technique of Biilmann (4) in which potassium ethyl xanthogenate is used was adopted.

*$\epsilon$ -Benzoylamino- $\alpha$ -Xanthogenate-*n*-Caproic Acid (II)*—A sample of 48 gm. (3 M) of potassium ethyl xanthogenate dissolved in 50 cc. of water was treated with 31.4 gm. (1 M) of  $\epsilon$ -benzoylamino- $\alpha$ -bromo-*n*-caproic acid, and the mixture shaken for about an hour in an *open* flask until in solution. The flask was stoppered and allowed to stand for 24 hours at room temperature. The solution was then shaken out once with ether to remove some oil, and 5 N HCl added until the reaction of the mixture was acid to Congo red paper. A brown oil appeared which after standing for several days in the ice chest crystallized. The material was filtered off, ground up in a mortar with water, filtered, and washed thoroughly with water. The yield of crude dried xanthogenate acid was 32 gm. or 94 per cent of the theory. It was crystallized twice from hot ethyl acetate. The substance separates from the solvent in short stout prisms and is slightly yellow in color. M. p., 112–114° to a clear yellow liquid.

$\text{C}_{16}\text{H}_{21}\text{NO}_4\text{S}_2$ (355.29).	Calculated.	N 3.94, S 18.1
	Found.	" 4.35, " 18.7



*ε-Benzoylamino-α-Thiol-n-Caproic Acid (III)*—A sample of 26.0 gm. of the xanthogenate acid was dissolved with warming in 150 cc. of absolute alcohol, cooled, and then treated with 100 cc. of 27 per cent  $\text{NH}_3$  solution in water. Enough alcohol was then added to bring the bulk of the solution nearly to the capacity of the flask. The latter was allowed to stand at room temperature for 3 days. The solution was then taken down to an acid-reacting syrup on the water bath, made slightly alkaline by addition of ammonia water, and shaken out twice with ether. To the cooled aqueous solution, concentrated  $\text{HCl}$  was added until the reaction was acid to litmus and then several pieces of  $\text{Zn}$  granules were added. The hydrochloric acid was now added further until the final reaction was acid to Congo red paper. A heavy oil separates which on continuous rubbing solidifies. The solid mass was broken up to a fine powder and allowed to stand in contact with the  $\text{Zn-HCl}$  mixture overnight in order to reduce any disulfide present. The mass is then filtered off, washed thoroughly with water, and then extracted several times with boiling absolute alcohol. The combined alcoholic extracts are filtered hot from the  $\text{Zn}$  particles and treated with hot water. The  $\epsilon$ -benzoylamino- $\alpha$ -thiol- $n$ -caproic acid crystallizes out rapidly on cooling in large white prisms. It is filtered off, washed with water, then with dilute alcohol, and dried. The yield was 14 gm. or 80 per cent of the theory. M. p.,  $158^\circ$  sharply to a clear liquid. It yielded a positive nitroprusside reaction.

$\text{C}_{15}\text{H}_{17}\text{NO}_2\text{S}$ (251.2).	Calculated.	N 5.57, S 12.6
	Found.	" 5.36, " 12.2

*ε-Amino-α-Thiol-n-Caproic Acid-HCl (IV)*—An amount of 95 gm. of  $\epsilon$ -benzoylamino- $\alpha$ -thiol- $n$ -caproic acid was dissolved in a liter of hot concentrated  $\text{HCl}$  and boiled under a reflux condenser for fully 12 hours. The solution was allowed to cool overnight, the benzoic acid filtered off, and the yellowish colored filtrate shaken out twice with ether. The aqueous solution was then evaporated *in vacuo* to a syrup, taken up in water several times, and evaporated each time to remove excess acid. The entire syrup plus a few crystals of unattacked benzoyl compound was taken up in about a liter of water, heated with norit, and filtered. The filtrate was then treated with 8 liters of saturated  $\text{HgCl}_2$

solution with stirring. A white oil first appears which in a few minutes turns crystalline. The mixture was allowed to stand overnight. The mercury salt is then filtered off, washed with dilute reagent, and dissolved with slight warming in 500 cc. of 10 per cent HCl solution. The latter was treated with  $H_2S$ , the mercuric sulfide removed with the aid of norit, and the clear filtrate evaporated *in vacuo* to a thick syrup. The latter is taken up several times in water to remove excess acid. On standing in the ice chest for several weeks the syrup becomes crystalline. With previously prepared seed crystals, complete crystallization is effected within an hour. The crystal mass is thoroughly dried *in vacuo* over NaOH. It is then dissolved in hot absolute ethyl alcohol, filtered, and treated with dry ether. On cooling, there crystallize large macroscopic prisms of the hydrochloride of  $\epsilon$ -amino- $\alpha$ -thiol-*n*-caproic acid. The yield amounted to 36 gm. The substance began to soften at  $117^\circ$  and melted to a clear liquid at  $123^\circ$ . The nitroprusside reaction was positive.

$C_6H_{14}NO_2S$ (199.62).	Calculated.	C 36.07, H 7.07, N 7.01, S 16.2
	Found.	" 35.92, " 6.99, " 7.03, " 16.9

$\epsilon, \epsilon'$ -Diamino-di( $\alpha$ -Thio-*n*-Caproic Acid) (V)—A sample of 35 gm. of the  $\epsilon$ -amino- $\alpha$ -thiol-*n*-caproic acid hydrochloride was dissolved in 240 cc. of water. With cooling, enough 10 per cent  $NH_3$  solution was added to make the mixture slightly alkaline, and then 3.5 cc. of  $FeCl_3$  solution (1:6). The color became deep purple-red. A stream of air was blown through the solution for 10 hours at the end of which time the purple color had disappeared. The solution was filtered and the filtrate evaporated *in vacuo* at a bath temperature of  $35^\circ$  to a syrup. The remainder of the drying occurred in a vacuum desiccator over  $P_2O_5$ . On alternate rubbing and decantation with absolute alcohol, the entire mass is transformed into a fine amorphous and hygroscopic powder. The material is suspended in 95 per cent alcohol and shaken on the machine for several hours to remove as much ammonium chloride as possible. The shaking is then repeated, 80 per cent alcohol being used with alternate decantation and renewal of diluted alcohol until the material yields a negative test for chloride ion. The material was then filtered off, washed with diluted alcohol, and then dissolved in the minimum amount of water. The latter

was shaken with norit, filtered, and to the clear colorless filtrate absolute ethyl alcohol was added in excess. An oil separates which after several days standing in the ice chest crystallizes into large feathery needles. The supernatant mother liquor is decanted and the precipitation procedure repeated two or three times. The amino acid is then filtered off, washed with alcohol and ether, and dried *in vacuo*. The yield of pure substance was 23.6 gm., or 84 per cent of the theory. M. p., 207° with decomposition. The compound is easily soluble in water due most probably to its highly polar nature. It is insoluble in organic solvents. The aqueous solution is acid to litmus. On warming the aqueous solution with basic lead acetate the molecule is disrupted and PbS is formed.

$C_{12}H_{24}O_4N_2S_2$  (324.31). Calculated. C 44.40, H 7.46, N 8.63, S 19.8  
Found. " 44.33, " 7.58, " 8.37, " 20.2

$\epsilon, \epsilon'$ -Diguanido-di( $\alpha$ -Thio-*n*-Caproic Acid)—An amount of 4.418 gm. of O-methylisourea hydrochloride (16, 20) in about 20 cc. of dry methyl alcohol solution was chilled in a freezing mixture and then 18.9 cc. of sodium methylate solution (2.11 N) were added. The sodium chloride was filtered off through a layer of infusorial earth and to the filtrate were added 2.78 gm. of  $\epsilon, \epsilon'$ -diamino-di( $\alpha$ -thio-*n*-caproic acid). The mixture was heated to about 50° and small portions of water added until in solution. The solution was filtered clear and placed in the ice chest. Crystallization of the guanidine derivative began after 24 hours and was apparently complete in 5 days. The yield was 1.5 gm. or 43 per cent of the theory. It was dissolved in a little hot water and treated with an excess of absolute ethyl alcohol. An oil appears which after standing in the ice chest for 48 hours crystallizes into aggregates of tiny needles. M. p., 178–180° with foaming. The aqueous solution of the guanido acid was alkaline to litmus. On prolonged boiling with basic lead acetate the molecule is disrupted, yielding PbS. Otherwise it is quite stable even in hot water. The acid yields a positive Sakaguchi reaction.

$C_{14}H_{28}O_4N_6S_2$  (408.40). Calculated. C 41.13, H 6.91, N 20.58, S 15.70  
Found. " 40.91, " 7.34, " 18.60, " 15.70  
" 18.42

The nitrogen analysis values are too low by approximately 10 per cent. In view of the excellent agreement between the theoretical and calculated values for the other elements, this discrepancy is inexplicable.

*ε,ε'-Diphenylureido-di(α-Thio-n-Caproic Acid)*—An amount of 1.62 gm. of *ε,ε'*-diamino-di(α-thio-*n*-caproic acid) was dissolved in 10 cc. of *N* KOH and the solution chilled in the ice bath. With continual shaking, 1.19 gm. of phenyl isocyanate were added in small portions. The solution was filtered and to the clear filtrate 5 *N* HCl was added to Congo blue. The phenylureido derivative crystallized out immediately. It was filtered off, washed thoroughly with large quantities of water, and then dried *in vacuo*. The yield amounted to about 95 per cent of the theory. The substance began to melt at 81°, then became solid above this temperature, and finally decomposed at 140°.

$C_{26}H_{34}O_6N_4S_2$ (562.3).	Calculated.	C 55.48, H 6.09, N 9.98
	Found.	" 55.24, " 6.09, " 10.38

#### SUMMARY

The syntheses of three new tetravalent amino acids have been described; namely, α-aminotricarballylic acid, m. p. 196°, α,γ,δ-triamino-Δ<sup>γ,δ</sup>-pentenic acid, m. p. 171–173°, and *ε,ε'*-diamino-di(α-thio-*n*-caproic acid), m. p. 207°. To further characterize the latter substance, its *ε,ε'*-diguanido derivative, m. p. 178–180°, and its *ε,ε'*-diphenylureido derivative, m. p. 140°, were prepared. The peptide of α-aminotricarballylic acid, namely glycyl-α-aminotricarballylic acid, m. p. 195°, was also prepared. The intermediate steps are described.

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# STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

## II. THE SYNTHESIS OF CERTAIN DERIVATIVES OF LYSYL-GLUTAMIC ACID

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The dipeptide, lysylglutamic acid (1), contains two amino groups and two carboxyl groups. The physicochemical properties of this tetrapole are elsewhere considered (4). The action of ereptic enzymes upon this compound has also been studied, as well as upon its benzoyl derivative (3). In the case of both substances, enzymic activity was relatively slow.

It would seem of interest to prepare further derivatives of this interesting dipeptide in which certain of the free groups are either masked or substituted. The present communication is concerned with the preparation of two derivatives of lysylglutamic acid, namely its diketopiperazine, anhydrolslylglutamic acid amide, and its  $\epsilon$ -guanido derivative,  $\epsilon$ -guanido- $\alpha$ -aminocapronylglutamic acid.

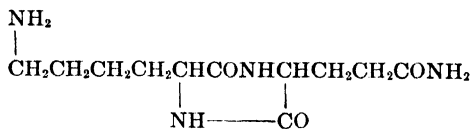
The investigations of Matsui (7) and Ishiyama (5) have indicated that activated trypsin slowly splits those diketopiperazines which contain one or more free carboxyl groups. The anhydrolslylglutamic acid amide is a diketopiperazine with a free  $\epsilon$ -amino group on the lysine residue of the ring, and a free  $\gamma$  acid amide group on the glutamic acid residue. Were diketopiperazines actually part of the structure of the protein molecule, many of the free groups of lysine and of glutamic acid might conceivably exist in the fashion indicated in the present anhydride model.

Up to the present time no peptides have been prepared containing arginine in the acyl position. The new method described by Bergmann, Zervas, and Rinke (2) for the introduction of arginine

into peptide combination has yielded glycylarginine in which arginine has been the second member of the dipeptide. The introduction of an  $\epsilon$ -guanido group into a dipeptide containing a diamino acid in acyl position such as in lysylglutamic acid would yield an arginine-like dipeptide derivative, differing from a true arginine peptide by possessing in the former case an extra  $\text{CH}_2$  group. The addition of a guanidine nucleus to the  $\epsilon$ -carbon atom of the lysine residue of lysylglutamic acid was easily accomplished by the procedure first applied to the amino acids by Kapfhammer and Müller (6). This method involves the use of O-methylisourea. Unfortunately the compound could not be obtained in crystalline form.

The investigation of the action of proteolytic enzymes on these and similar peptide derivatives will be reported in a later communication.

*Anhydrolysylglutamic Acid Amide*



The lysylglutamic acid employed in this and the following synthesis of the  $\epsilon$ -guanido derivative was prepared according to the method of Bergmann, Zervas, and Greenstein (1).

#### EXPERIMENTAL

2 gm. of lysylglutamic acid- $2\text{H}_2\text{O}$  are suspended in 20 cc. of absolute methyl alcohol, the mixture chilled in an ice bath, and then dry  $\text{HCl}$  gas passed through the mixture to saturation. The solution was evaporated *in vacuo* at a low temperature to a syrup, the latter taken up in methyl alcohol, and the esterification and subsequent evaporation *in vacuo* repeated. Inasmuch as the ester hydrochloride syrup failed to crystallize, it was taken up in the minimum amount of dry methyl alcohol and carefully added with chilling to 40 cc. of dry methyl alcohol saturated at  $0^\circ$  with dry ammonia gas. The entire solution is allowed to stand for 5 days in the ice box, at the end of which time maximal crystallization of the diketopiperazine derivative in the form of tiny needles

had occurred. The substance was filtered off, washed with alcohol and ether, and dried *in vacuo*. The yield amounted to 1.0 gm. or about 60 per cent of the theory. The melting point of the crude substance was 226–235° with charring. It was dissolved in the minimum amount of water, filtered, and the filtrate treated with an equal volume of absolute alcohol and then with an excess of dry ether. An oil appeared which after standing overnight in the ice chest crystallized into needles. The material was filtered off, washed, and dried *in vacuo*. The yield of pure substance was now 0.65 gm. It melted sharply at 242°. The aqueous solution was slightly alkaline to litmus and on addition of strong alkali evolved  $\text{NH}_3$ .

$\text{C}_{11}\text{H}_{21}\text{N}_4\text{O}_5\text{Cl}$ (292.66).	Calculated.	C 45.20, H 7.23, N 19.14
	Found.	" 45.55, " 7.32, " 18.90

*ε-Guanido-α-Aminocapronylglutamic Acid*—Attempts to prepare homologues of arginine were reported by Winterstein and Kung (8). The latter used cyanamide in the addition reaction on the end-standing amino groups of  $\alpha$ ,  $\beta$ -diaminopropionic acid and of lysine. They were successful in forming a  $\beta$ -guanido- $\alpha$ -aminopropionic acid but failed to prepare the corresponding guanido derivative of lysine. The strongly basic properties of these molecules would be expected to inhibit the reaction with cyanamide. As indicated below in the present reaction of lysylglutamic acid with O-methylisourea, only one guanidine group is introduced in the molecule. Boiling the monoguanidopeptide with concentrated HCl hydrolyzes the peptide bond, yielding in the case of an  $\alpha$ -guanido-substituted lysine residue the corresponding creatinine analogue, anhydro- $\alpha$ -guanido- $\epsilon$ -aminocaproic acid. In the case of an  $\epsilon$ -guanido-substituted lysine residue the hydrolysis products would yield the unaltered  $\epsilon$ -guanido- $\alpha$ -aminocaproic acid as the hydrochloride salt. In the former case the Sakaguchi reaction would be negative, in the latter case positive. On treating lysylglutamic acid with excess of O-methylisourea there is formed a monoguanidomonoaminocapronylglutamic acid which yields, as would be expected, a positive Sakaguchi reaction. After boiling with concentrated HCl, the hydrolysis mixture yielded again a strongly positive Sakaguchi reaction, indicating that the guanido group was on the  $\epsilon$ -carbon atom of the lysine residue.

A solution of 2.8 gm. of O-methylisourea in 20 cc. of absolute



methanol was treated with 2.3 gm. of lysylglutamic acid. The mixture was then shaken at room temperature until complete solution occurred and then set in the ice chest. After 48 hours an excess of absolute alcohol was added, resulting in the separation of a dense flocculent precipitate. The latter was filtered off and washed rapidly with alcohol and ether as it is somewhat hygroscopic. After complete drying in a vacuum it is no longer hygroscopic. The yield was rather low, amounting to only 0.9 gm. The substance was dissolved in the minimum amount of cold water, filtered, and to the filtrate absolute alcohol added in excess. An oil separates which after standing in the ice chest for several days solidifies. The yield sinks following this procedure to 0.12 gm. The melting point was 95° with foaming. The aqueous solution was quite alkaline to litmus, gave a positive Sakaguchi reaction, and yielded a dense precipitate with phosphotungstic acid. A sample of 0.10 gm. was boiled with 10 cc. of 5 N HCl for 3 hours. At the expiration of this time it was evaporated to a syrup *in vacuo* to remove as much HCl as possible. The residue yielded a strongly positive Sakaguchi reaction.

$C_{12}H_{23}O_5N_5$  (317.2). Calculated. C 45.39, H 7.31, N 22.10

Found. " 45.12, " 7.71, " 22.38

Calculated for diguanidocapronylglutamic acid: C 43.43, H 7.01, N 27.28

#### SUMMARY

The preparation of two derivatives of the dipeptide lysylglutamic acid is described. These derivatives are anhydrolsylv-glutamic acid amide as the hydrochloride salt, m. p. 242°, and  $\epsilon$ -guanido- $\alpha$ -aminocapronylglutamic acid, m. p. 95°.

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## CYSTINURIA\*

### III. THE METABOLISM OF SERINE

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Although the cystinuric individual can completely oxidize orally administered cystine, it has been shown recently<sup>1</sup> that cysteine and methionine are only partially oxidized in this disease. The greater part of the sulfur of the latter two amino acids appears in the urine as extra cystine. These findings were interpreted as indicating that one of the pathways of methionine metabolism is its conversion into cysteine, the first step being probably a demethylation to homocysteine. More insight into the complicated mechanisms involved could be gained only by further experiments.

In view of the close structural relationship between cysteine ( $\beta$ -thiol- $\alpha$ -aminopropionic acid) and serine ( $\beta$ -hydroxy- $\alpha$ -aminopropionic acid), it was of interest to investigate the fate of the latter amino acid<sup>2</sup> in the cystinuric organism. It will be seen from the data presented below that the feeding of *dl*-serine or of *dl*-serine plus *l*-cystine did not alter the urinary cystine excretion.

#### EXPERIMENTAL

The metabolic observations were carried out on the same patient and in the same manner as previously described.<sup>1</sup>

It can be seen from Table I that the nitrogen, sulfur, and

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<sup>1</sup> Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935).

<sup>2</sup> Serine does not promote growth of rats upon a diet deficient in cystine; cf. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **98**, 465 (1932).

cystine excretion in control Periods 15 and 16 resemble closely the control periods reported in the preceding publication.<sup>1</sup> The administration of 4 gm. of *dl*-serine (Period 15-a) and of 2 gm. of *dl*-serine plus 3.2 gm. of cystine (Period 15-b) did not change the daily excretion of cystine as determined by the Folin and the

TABLE I  
*Metabolic Observations*

Period No.	Date	Substance fed		Urine									
				Nitrogen			Creatinine		Sulfur			Cystine	
				Total	Urea	Amino acid	Preformed	Total	Total	Inorganic SO <sub>4</sub>	Ethereal SO <sub>4</sub>	Folin	Sullivan
	July, 1933		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
15*	9-14			5.9	4.2	0.22	1.07	1.29	0.56	0.26	0.05	0.72	0.62
15-a	15	Serine	1	5.9	4.9	0.15	1.07	1.34	0.51	0.22	0.04	0.71	0.60
	16		1	5.9	4.5	0.15	1.10	1.32	0.53	0.22	0.07	0.73	0.60
	17		2	5.7	4.3	0.16	1.07	1.26	0.49	0.20	0.04	0.69	0.58
15-b	18	Serine + cystine	2	6.2	4.2	0.15	1.03	1.34	1.02	0.70	0.04	0.62	0.57
			3.2										
15-c	19			5.6	4.2	0.16	1.12	1.34	0.61	0.32	0.05	0.72	0.60
	20			5.7	4.2	0.15	1.09	1.32	0.59	0.29	0.05	0.70	0.58
16*	21-24			5.5	3.9	0.12	1.08	1.22	0.52	0.22	0.05	0.71	0.60

\* In these periods figures indicate average daily excretion.

Sullivan methods. It may be noted also that the rise in inorganic sulfate following the ingestion of serine plus cystine is not different from that observed in the previous experiments with cystine alone.

#### SUMMARY

1. Serine and serine plus cystine were fed to a cystinuric patient.
2. The level of cystine excretion remained unchanged during the feeding periods.

# THE VAPOR PRESSURE OF HUMAN BLOOD BY HILL'S THERMOELECTRIC METHOD. APPARATUS AND TECHNIQUE

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In 1930 Margaria (1) determined the normal vapor pressure of adult defibrinated blood, using the thermoelectric method suggested by Hill (2). Hill's description of the apparatus, while presented in some detail, is not readily applicable to the construction of the instrument from materials obtainable in this country. Moreover, it was found by experience that a number of important points, not previously stressed, must be kept in mind to insure the building of a workable thermopile. It is felt that concise information as to materials and technique will be of some value and the details here introduced represent the instrument as constructed in this laboratory and used in the present experimental work.

The present report deals with the application of this method to the determination of normal values of vapor pressure for infants' and children's blood, there being no previous reports of this nature as far as can be determined. In a somewhat related field Gilman and Cowgill (3) have shown that simultaneously collected dog blood, hepatic bile, pancreatic juice, and lymph are practically isotonic, while artificially produced variations in blood osmotic pressure result in parallel changes in these fluids. Again, Gilman and Yudkin (4), using the same thermoelectric method, noted that the aqueous humor is isotonic with the blood of the dog. Grollman (5) applied this technique in a form slightly modified so as to increase its sensitivity, in his studies of the vapor pressure of mixed aqueous solutions with reference to the state of water in biological fluids.

The method depends upon the difference in temperature attained by opposite faces of a symmetrically wound thermopile when on

one face is a filter paper saturated with the defibrinated blood while on the other is placed a similar piece of filter paper soaked in a standard sodium chloride solution. The temperature difference resulting from the unequal rates of evaporation from the two faces produces an E.M.F. proportional to this difference, which is read on a sensitive galvanometer. The thermopile having been previously calibrated with NaCl solution of accurately known concentration, the vapor pressure of the blood can be calculated in terms of the standard solution.

### *Apparatus*

As shown in Fig. 1, the apparatus consists of a thermopile wound on an insulated brass frame and screwed into the cover of a cylindrical brass case, the whole being attached to a heavy brass tube about 2 feet long which carries the lead-in wires from the copper terminals supported by the hard rubber base fitted to the upper extremity of the tube. This tube *B* with an outside diameter of  $\frac{3}{8}$  inch and inside diameter of  $\frac{1}{4}$  inch serves also for the exit of gas from the chamber *C*, formerly a microscope objective case of 25 cc. capacity. The small brass tube *A*, running parallel to *B* and fastened to the latter for support, also passes through the cover; it is  $\frac{1}{8}$  inch in outside diameter and serves for the admission of gas to the chamber, an appropriate stop-cock being attached at its upper end when in use. The thermopile frame *E* is constructed of  $\frac{1}{8}$  inch square brass rods so that the outside dimensions are  $\frac{3}{4}$  inch by 1 inch. It was found that this size frame cleared the wall of the chamber by about 2 mm. on either side when the instrument was complete and insulated ready for use. The four elements composing the frame may be fastened together in the most convenient way, *i.e.* dovetail, countersunk screws, hard soldering, etc., the only essential feature being solidity of construction. To the frame *E* is attached a small piece of brass tubing,  $\frac{1}{4}$  inch outside diameter and  $1\frac{1}{2}$  inches long. This short tube serves as a convenient handle for the thermopile during subsequent treatment and winding. As may be seen in Fig. 1, its free end is threaded to the heavy brass tube, into which it is finally screwed when the instrument is assembled. Thus the thermopile is held mechanically firm at all times. Two  $\frac{3}{32}$  inch holes are drilled through the short tube near its upper threaded portion, as shown, one of which serves for the

entrance of the lead-in wires *D* to the chamber, while the other serves as a gas outlet as well as for equalizing the pressure of gas within the case with that of the atmosphere when the instrument is in operation. A  $\frac{1}{16}$  inch hole *F* through the bottom of the thermopile permits equalization of the gas pressure within the winding with that of the chamber. This hole must be kept patent at all times during construction, as well as when the instrument is in use, for expansion of confined gas within the winding would

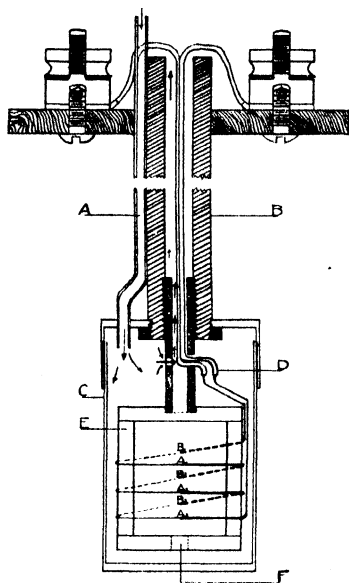


FIG. 1. Schematic section showing thermopile in brass container

blow out the varnish coating, to be described below, and ruin the thermopile. The dimensions suggested here represent only the size found most convenient for the type of experiment reported; they may well be varied to suit other work. It may be said in general, however, that larger instruments and containers offer no advantage from the standpoint of accuracy and that they require a longer time for equilibration in the constant temperature bath, besides requiring more fluid to cover their faces. In fact, as has been pointed out by Grollman, sensitivity may be increased by

decreasing the distance between face and wall, since there is a gradient of vapor pressure between the solution on the wall and those on the faces. Because proximity increases this gradient and causes a more active exchange by evaporation or condensation at the thermopile faces there results a greater temperature difference between the two faces with a correspondingly increased E.M.F.

The brass frame, having been previously cleaned and smoothed down, preferably on a buffing wheel, is covered with a thin coat of No. BV-1307 bakelite varnish which has been properly thinned to the consistency of light machine oil with No. BV-1030 bakelite thinner. Brushing on the varnish produces a more evenly distributed coating than dipping. The instrument is then allowed to dry in a dust-free atmosphere for 4 to 8 hours, after which it is placed in an oven for baking. The oven is gradually brought to a temperature of 85° and is maintained at this level for  $\frac{1}{2}$  to  $\frac{3}{4}$  hour when the temperature of the oven is raised to 130°, where it is kept for  $\frac{1}{2}$  hour longer. Then the oven is turned off and the instrument allowed to return to room temperature slowly, for rapid cooling may crack the varnish coating, which is made very hard by the baking process. Five or six coats of varnish are necessary, each one being baked in the same manner as the first. When this is done, the brass frame will be covered with a hard, effective insulator which, at the same time, is not thick enough to modify greatly the heat-conducting quality of the brass.

The frame, satisfactorily varnished, is now ready for winding. Two pieces of thin high grade paper are lightly shellacked, dried, and then placed along the sides of the frame upon which the winding is to rest. They may be held in place temporarily by small rubber bands until a turn or two of the wire is wound. These papers act as a protection to the varnish, preventing the wire from cutting through with the possibility of short circuit. A No. 36 Brown and Sharpe gage bare constantan wire of highest quality is used, which may be obtained from Leeds and Northrup Company. An appropriate anchorage, such as a double half-hitch of wire, is made at the upper end of the frame and the winding is started either by hand or on a lathe. The turns are placed as close together as possible, without touching; there will be about 60 in all. The tensile strength of the thin wire is not very great, but it is

important that the tension on the wire be kept constant throughout the winding, for during later heat treatment much distortion occurs if the tension has been varied appreciably. It may be mentioned that the handling of the wire is best accomplished with gloves; otherwise the grease and oil which accumulate will be difficult to remove without damage to the coil. A chemically clean wire is necessary for plating. In order to insure a clean, shining wire, a double silk-covered No. 36 Brown and Sharpe gage constantan wire may be stripped of its insulation by gently rubbing about 20 feet of the stretched wire with No. 000 sandpaper, while wearing gloves, and then rewinding it back on the spool. The wire, with its freshly exposed surface, is free of troublesome oxide film and takes the silver plate evenly.

The ordinary double silver cyanide plating solution (6), somewhat diluted, gives very satisfactory results. It may be prepared as follows:

To 500 cc. of a saturated solution of silver nitrate, freshly prepared, saturated sodium cyanide solution is added until no further precipitation occurs, care being taken that excess NaCN is not added. The precipitated silver cyanide,  $\text{AgCN}$ , is washed several times with distilled water by decanting, and NaCN is added again in sufficient amount to dissolve the precipitate, solution occurring because of the formation of the complex argenticyanide ion,  $\text{Ag}(\text{CN}_2)^-$ , to which the desirable properties of the plating bath are in large measure due. Excess NaCN, amounting to about one-third the total volume of the solution, may now be added; it facilitates plating and maintains the anodes clean and white. Finally, the solution is diluted to 1600 cc. with distilled water and allowed to stand 24 hours before being used. In this diluted state there is no noticeable action of the cyanide on the baked varnish exposed to it for 1 hour. The varnish is rapidly softened by commercial silver plating cyanide baths.

The anodes, two in number, and about 3 inches square, may be of thin sheet silver obtainable from Eimer and Amend. Should they become discolored during plating or from exposure to air, they may be cleaned by dipping in saturated NaCN solution or by rubbing the solution over their surfaces with gauze compresses. The discoloration usually means that there is insufficient NaCN in the plating bath, but large excess must be avoided for the reason



given. Slight discoloration of the anodes does not interfere with the plating.

A large specimen jar about 10 inches in diameter and 4 inches deep makes a suitable plating tank. The thermopile is so suspended that one-half of each turn of wire will be plated, *i.e.* with the long axis of the instrument horizontal and the plane of the coil at right angles to the surface of the bath. The line of plate on each face must be at the mid-line, parallel to the long axis of the instrument. The latter is rigidly supported, as are also the anodes, which are placed opposite each face and as far away from the thermopile as possible. It is important that no rippling of the liquid occur, since the line of juncture between the plated and the unplated portion must be distinct. Therefore all connections are made before the plating solution is poured into the jar. A 500 ohm rheostat of the continuously variable type, together with a milliammeter and switch, are placed in series with the positive terminal of a fully charged 6 volt storage battery and the anodes. A piece of copper foil is snugly attached all along the upper border of the winding as well as to the thermopile leads and thence to the negative terminal of the battery. This insures a uniform deposition of silver. The solution is introduced from a funnel and connecting tube directly into the bottom of the tank by means of a controlling stop-cock, so as to regulate the height to which the liquid rises and at the same time avoid rippling of the bath. It is easier to judge the point where the solution meets the center of the instrument if the thermopile has been previously marked. Should the solution get on any portion of the unimmersed wire, silver will be deposited. This is obviously undesirable. A current of from 4 to 6 milliamperes is allowed to flow for about  $\frac{1}{2}$  hour and a smooth white deposit of silver results. Excess current causes the emission of bubbles near the ends of the coil and produces a loose, irregular deposit of silver with discoloration of the plate. In this event the thermojunctions will not be distinct. Therefore the entire 500 ohms should be included in the circuit at the start and the proper current value determined by observing the manner of deposition of the plate. After plating, the instrument is washed with distilled water until the last trace of cyanide has been removed. If the instrument is removed from the bath in exactly the same relative position which it held while immersed, and

water is gently poured over it, the stream will flow from the dry toward the plated portion, thereby preventing the cyanide solution from creeping upward. The instrument may be dipped into a beaker of water after the first washing has been completed. After drying overnight at room temperature, the last traces of moisture are removed by heating in a thermostat at  $70^{\circ}$  for 1 hour. The thermostat should be brought up to  $70^{\circ}$  gradually and after the completion of drying allowed to cool slowly. To avoid oxidation of the silver plate varnishing of the instrument should not be delayed.

The varnishing of the plated thermopile is accomplished in the same manner as that of the frame, as previously described. The varnish is applied with a soft camel's-hair brush, avoiding excess, and the instrument allowed to dry as before. It is advisable when baking to raise the oven temperature a little more slowly than previously to  $85^{\circ}$ , followed by baking at  $130^{\circ}$  for  $\frac{1}{2}$  hour. After five or six coats have been applied the thermopile will be imbedded in a smooth translucent matrix of varnish with no intervening free spaces between the turns.

The instrument is then screwed into the heavy brass tube and the thermopile leads soldered to the rubber-covered copper terminal wires. These terminal wires must be free from tin coating such as is often found on insulated wire. The thermopile, including the soldered leads, is next immersed in a mixture of equal parts of paraffin and beeswax at  $85^{\circ}$ . The wax is allowed to drain off; with a little practise a smooth thin coating results. The instrument is reparaftined at 4 to 8 day intervals depending upon the extent to which it is used. The small hole in the bottom of the frame must be kept patent at all times for the reasons previously mentioned. One or two coats of varnish are baked onto the interior of the chamber to avoid electrolytic action when filter paper soaked in NaCl solution is placed on the wall.

#### *Theory and Method of Calibration*

The theory of operation has been thoroughly presented by Hill (7) and Margaria (1) and is appended here merely for the sake of completeness. Suppose that on face *A* of the thermopile is placed a filter paper moistened with a solution *a*, while on face *B* is placed a similar filter paper with a solution *b*, and on the wall of

the chamber is a large filter paper moistened with a solution  $c$ . Let the vapor pressures of the solutions be represented by  $P_a$ ,  $P_b$ , and  $P_c$ , respectively. Then the predominating vapor pressure in the chamber will be  $P_c$  except in the immediate neighborhood of faces  $A$  and  $B$ , where the vapor pressures will be those respectively of the solutions  $a$  and  $b$ . The rate of evaporation from or condensation on face  $A$  will be  $K(P_c - P_a)$ ; that for face  $B$ , similarly  $K(P_c - P_b)$ . When equilibrium is finally reached at constant temperature, the difference in temperature of the two faces will be represented by  $K'(P_c - P_a)$  for face  $A$  and for face  $B$  by  $K'(P_c - P_b)$ .  $K'$  is a constant dependent on barometric pressure, temperature, thermal conductivity, and design of instrument, etc.; it will be different for each thermopile. At equilibrium, then, the difference in temperature between the two faces will be  $K'(P_b - P_a)$ , which is independent of  $P_c$  for a perfectly symmetrical instrument. However,  $K'_{\text{face } A}$  does not equal  $K'_{\text{face } B}$  in practise, for it would be very difficult to build a perfectly symmetrical thermopile; so that the temperature difference between the two faces is

$$K'_A (P_c - P_a) - K'_B (P_c - P_b)$$

If the solutions on the thermopile faces are reversed in positions while the wall filter paper is left unchanged, and another determination is made, the effect due to asymmetry is eliminated, for the mean of the two determinations may be calculated to be

$$\frac{K'_{\text{face } A} + K'_{\text{face } B}}{2} (P_a - P_b)$$

where  $P_c$  again cancels out.

Actually, calibration is carried out by placing 0.92 per cent NaCl<sup>1</sup> solution on the chamber wall and also on one face of the thermopile, while on the other is a filter paper moistened with distilled water. The instrument is placed in a large constant temperature water bath at approximately 25°, controlled accurately to 0.001° by a vacuum tube relaysystem, and allowed to attain equilibrium. This requires about 20 minutes. A double pole-double throw switch, interposed between the galvanometer and the thermo-

<sup>1</sup> The term 0.92 per cent NaCl always means a solution containing 0.92 gm. of dried sodium chloride per 100 gm. of distilled water.

pile, allows the direction of the current to be reversed and helps to eliminate the effects of parasitic E.M.F. which may be troublesome in a circuit of this kind. The sum of two switch reversals is noted (making one reading). Then the readings are repeated with the solutions on the faces reversed and the mean of the two readings is computed. This constitutes one double observation. The con-

TABLE I  
*Calibration of Thermopiles*

The water bath was at  $25^{\circ} \pm 0.001^{\circ}$ ; chamber walls, 0.92 per cent NaCl.

Thermopile No.	Face A	Face B	Galvanometer deflection	Mean of two reverse readings	Equivalent gm. per cent NaCl per mm. deflection
7	0.92% NaCl	H <sub>2</sub> O	mm.	492.3	0.00187
			-478.3		
			-480.7		
			-480.1		
			479.6		
	H <sub>2</sub> O	0.92% NaCl	+506.0		
8	0.92% NaCl	H <sub>2</sub> O	+505.0	477.2	0.00193
			+504.0		
			+504.0		
			505.0		
			-478.2		
			-476.1		
			-476.3		
			476.8		
	H <sub>2</sub> O	0.92% NaCl	+476.8		
			+477.9		
			+478.2		
			477.6		

centration of NaCl per mm. of deflection of the galvanometer is readily obtained from this mean. The reversing switch is always closed in the same way and the leads from the switch to the thermopile, as well as the thermopile terminals, are so labeled that the same lead is always connected to its corresponding terminal on the instrument. In this way the initial direction of current through the galvanometer is established during calibration, for comparison

with the initial direction when the vapor pressure of a blood sample is observed, thereby indicating whether to add or subtract the value for the blood sample to or from the standard solution. An examination of Table I will make clear the method of calibra-

TABLE II  
*Readings on Blood Sample*

The water bath was  $25^{\circ} \pm 0.001^{\circ}$ ; chamber walls, 0.92 per cent NaCl.

Thermo- pile No.	Face A	Face B	Gal- vanometer deflection	Mean of two reverse readings	Mean deflection, gm. per cent NaCl	Vapor pressure of blood sample as equivalent gm. per cent NaCl
			<i>mm.</i>	<i>mm.</i>		
7	0.92% NaCl	Blood	-7.0	13.7	0.0256	0.9236
			-7.0			
			-9.0			
			<u>7.6</u>			
	Blood	0.92% NaCl	+20.0			
			+19.6			
<u>+20.1</u>						
19.9						
8	0.92% NaCl	Blood	-11.9	11.2	0.0216	0.9236
			-11.9			
			-11.2			
			<u>11.6</u>			
	Blood	0.92% NaCl	+11.0			
			+11.1			
<u>+10.6</u>						
10.9						

tion; Table II shows the procedure used in obtaining readings on a blood sample.

The instruments were calibrated daily but before a thermopile was considered sufficiently accurate for blood work, blank tests were made by using a solution nearly isosmotic with the standard and then calculating its concentration from the galvanometer deflections. Thus, a 0.90 per cent NaCl solution was determined

opposite the standard 0.92 per cent NaCl. The instruments conformed to an allowable error of 0.5 per cent.

The galvanometer used was a Zernike moving coil, magnetic shunt, type D, manufactured by Kipp, Delft, Holland. It has a sensitivity of 1.0 to 0.25 microvolt per mm. of deflection at 1 meter, and when set at one-half maximum sensitivity may be read easily to 0.2 mm. on a scale at 1 meter.

The standard, containing 0.92 gm. of dried NaCl (Kahlbaum) per 100 gm. of water, was prepared in large amount. (In a few of the earlier experiments the standard solution contained 0.90 gm. of NaCl per 100 gm. of  $H_2O$ .) The water content of these solutions was checked by drying and weighing in the usual manner, and in addition specific gravity determinations were made. When the solution was freshly prepared, its specific gravity was determined accurately at 25° with 50 cc. pycnometers. Thus, a convenient and accurate method was at hand for checking any variations in concentration of the standard solution. During the course of the work no significant deviation from the first specific gravity reading was observed.

The blood was drawn from the median cubital vein in older children and from the external jugular in the case of infants. It was transferred to a small bottle containing glass beads and shaken to defibrinate. 2 cc. were found sufficient for four double observations. The appropriate size of filter papers, sufficient to cover the faces of the thermopile leaving about a 2 mm. margin all around, was either punched out with a steel die or cut in large numbers on a paper cutter. The large filter papers, for lining the chamber completely, were prepared likewise. A filter paper soaked in, but not containing an excess of, the freshly drawn defibrinated blood was placed on one face, while a similar filter paper, saturated with the standard 0.92 per cent NaCl solution, was laid smoothly on the opposite face of the thermopile, care being taken to avoid small air inclusions between the face and filter paper. By having the wall filter paper wet with 0.92 per cent NaCl also, very little change in concentration occurs in any of the fluids when dynamic equilibrium is established. The thermopiles were placed in the constant temperature bath at 25° and when equilibrium was established in 20 minutes the galvanometer deflections were recorded.

As was shown by Margaria (1), it is essential that the  $\text{CO}_2$  tension of the blood samples be kept constant, if comparable results are to be obtained. Unless this is done, discrepancies appear which are well outside the limit of error of the method. Therefore, a gas mixture of 5.0 per cent  $\text{CO}_2$  and 95 per cent  $\text{O}_2$  was saturated with moisture at the temperature of the bath and then introduced into the thermopile chamber through tube *A*, Fig. 1. This gave a  $\text{CO}_2$  tension about equal to that of arterial blood. When sufficient gas had passed through the system (about 250 to 300 cc.) tube *A* was closed with a stop-cock, tube *B* being left open. Thus, while convective currents were practically eliminated, the interior of the chamber was at atmospheric pressure. The gas mixture was analyzed in a Haldane apparatus at intervals during the course of the experiment and averaged between 4.5 and 5.2 per cent  $\text{CO}_2$ —an allowable variation for purposes of this work. The  $\text{CO}_2$  sample for analysis was drawn from the system at the point where the gas entered the thermopile.

When the 5.0 per cent  $\text{CO}_2$  gas mixture is introduced into the chamber, it is dissolved by the 0.92 per cent  $\text{NaCl}$  in an amount corresponding to its partial pressure.<sup>2</sup> In terms of an osmotically equivalent quantity of sodium chloride, this amounts to 0.0036 gm. of  $\text{NaCl}$  (8). The value, 0.0036 gm., is therefore added to the 0.92 per cent  $\text{NaCl}$  as a  $\text{CO}_2$  correction, making the standard solution value 0.9236 gm. of  $\text{NaCl}$  per 100 gm. of  $\text{H}_2\text{O}$ , and all values for blood samples are calculated from this value.

While it is not strictly true that blood drawn from the vein aerobically, defibrinated, and then subjected to 5.0 per cent  $\text{CO}_2$  represents the actual osmotic relation existing in capillary blood, it may be assumed for purposes of the experiment that it approaches

<sup>2</sup> The partial pressure of  $\text{CO}_2$  dissolved in 0.92 gm. per cent (0.157 M)  $\text{NaCl}$  is calculated from data given in the "International critical tables," volume 3. It amounts to 36.976 mm. of Hg at 25°. The volume *V* of  $\text{CO}_2$  absorbed is then obtained from the expression,  $V = avp/760$ , where *a* equals the absorption coefficient of 0.157 M  $\text{NaCl}$  at 25°, *v* equals 100 (gm.) of solution, and *p* is the partial pressure of  $\text{CO}_2$  given above. Thus

$$V = \frac{(0.736)(100)(36.976)}{760}$$

= 3.580 cc., or 0.0070 gm. at s.p.t.  $\text{NaCl}$  in 0.15 M concentration is about 94.2 per cent ionized; therefore the osmotic equivalent of 0.0070 gm. of  $\text{CO}_2$  in terms of  $\text{NaCl}$  is  $(1/1.942)(0.0070)$ , or 0.0036 gm. of  $\text{NaCl}$ .

this condition approximately; but what is more important, it permits of a standardized technique in handling blood samples in air and yet subjecting them to constant environmental conditions before measurements are made. When measurements are made on the same sample of blood in both the equilibrated and unequilibrated state, it is found that in the latter instance the values are, in general, lower by the osmotic equivalent of about 0.03 gm. per cent NaCl. Therefore, it is probable that when the blood sample is subjected to a 5.0 per cent CO<sub>2</sub> tension, there occurs an increase in the amount of total osmotically active substances, principally as a result of the CO<sub>2</sub> effect on the hemoglobin. The shift of anions from cells to plasma and *vice versa*, as well as the water shift to maintain the osmotic equilibrium seems, in this case, to be largely a function of the amount of hemoglobin present in the sample, the total fixed base remaining constant. In fact, it was noted in the few cases in which the hemoglobin was high that the vapor pressure values were in the upper limits of the normal, although there was no reason to suspect abnormality of the total electrolyte otherwise.

### Results

In order to appraise the accuracy, or better the reproducibility, of figures obtained by the method described in this paper, the probable error of measurement has been calculated. 50 analyses, performed in triplicate, *i.e.* 150 determinations, were utilized in the calculation. The separate determinations on each sample were divided among three columns in the order in which the measurements were made, so that each column contained 50 figures. The coefficient of reliability ( $r$ ) was then computed between the first and second columns, between the second and third columns, and between the first and third columns by means of the formula<sup>3</sup>

$$r = \frac{\sum xy}{\sqrt{\sum x^2} \sqrt{\sum y^2}}$$

in which  $x$  and  $y$ , respectively, refer to deviations from the average value for each column. The three  $r$  values so obtained were in

<sup>3</sup> The formulas used in the statistical treatment of the data are taken from Garrett (9).



close agreement. Their average value was used for calculating the probable error of measurement from the formula  $P.E.(M) = 0.6745\sigma\sqrt{1-r}$  the  $\sigma$  being the average of the standard deviations of the three columns. The  $P.E.(M)$  so obtained was 0.0051 gm. per cent NaCl, which means that the chances are even that any single determination will not differ from the theoretical mean of an infinity of determinations on the same sample by more than this amount. Furthermore, the chances are 993 in 1000, *i.e.* practically certain, that a single determination will not differ from the true mean by more than 4 times this amount or 0.0204 gm. per cent NaCl.

In practise it will rarely happen that a single determination only will be made on a sample. More commonly the analysis will be run in duplicate and the average of the two determinations accepted. In the study of the blood of 50 normal children, to be discussed in a moment, multiple determinations were made on each sample as follows: three determinations per sample eleven times; four determinations per sample thirty-seven times; five determinations per sample two times. It is obvious that the probable error of measurement will be decreased when the analytical result represents the average of two or more determinations. The change can be calculated by first computing the effect of multiple determinations on the reliability coefficient. This is done with Spearman's "prophecy" formula

$$r_x = \frac{Nr}{1 + (N-1)r}$$

in which  $N$  represents the number of determinations which have been averaged to arrive at a result. By substituting  $r_x$  for  $r$  in the formula previously given for  $P.E.(M)$  a new probable error of measurement will be obtained which expresses accurately the reproducibility of analyses which depend upon the average of several determinations. The calculation has been made for the vapor pressure method here described and discloses the following probable errors of measurement.

	NaCl gm. per cent
P.E.(M) (single determination).....	0.0051
" (average of two determinations).....	0.0038
" ( " " three " ).....	0.0031
" ( " " four " ).....	0.0027

TABLE III

*Vapor Pressure of Blood in Terms of Gm. of NaCl per 100 Gm. of Water*

Sample No.	Sex	Age	Vapor pressure	Sample No.	Sex	Age	Vapor pressure
		years	gm. per cent NaCl				gm. per cent NaCl
1	M.	4	0.9065	26	M.	6 yrs.	0.9142
2	"	4	0.8946	27	"	11 "	0.9249
3	F.	11	0.8541	28	"	6 mos.	0.9011
4	"	9	0.8929	29	"	3 yrs.	0.9003
5	"	5	0.9092	30	"	11 "	0.9132
6	"	8	0.9050	31	"	8 "	0.9346
7	"	3	0.8927	32	"	7 "	0.9014
8	"	9	0.9006	33	"	4 "	0.9076
9	M.	6	0.9044	34	F.	11 "	0.9150
10	F.	2	0.9097	35	"	1 yr.	0.9287
11	"	3	0.9298	36	"	3 yrs.	0.9070
12	"	2	0.9065	37	M.	9 mos.	0.8707
13	M.	10	0.8885	38	F.	5 yrs.	0.9087
14	"	11	0.8856	39	M.	7 "	0.9071
15	"	12	0.9101	40	"	11 "	0.9009
16	"	5	0.9058	41	"	5 "	0.9120
17	F.	10	0.9315	42	"	1 mo.	0.8969
18	M.	11	0.9142	43	F.	6 mos.	0.8792
19	F.	10	0.9274	44	M.	6 "	0.8849
20	"	5	0.9127	45	F.	11 "	0.9034
21	"	9	0.9045	46	M.	11 "	0.8627
22	M.	8	0.9071	47	F.	2 yrs.	0.9145
23	"	6	0.9268	48	M.	6 "	0.8843
24	"	3	0.8795	49	"	9 mos.	0.9063
25	F.	2	0.8951	50	"	1 yr.	0.8705

Average (50 samples) = 0.9029 gm. per cent NaCl. Standard deviation of distribution = 0.0171 gm. per cent NaCl. Probable error of average = 0.0016 gm. per cent NaCl.

TABLE IV

*Vapor Pressure Measurements*

	No. of determinations	Average	Standard deviation	Probable error (average)
Children	30, boys	0.9006	0.0168	0.0020
	20, girls	0.9053	0.0172	0.0026
Adults (Margaria (1))	19, men	0.9447	0.0074	0.0011
	16, women	0.9269	0.0088	0.0015

The findings in this investigation on the blood of 50 normal children whose ages ranged from 1 month to 12 years are shown in Table III. The average value was 0.9029 gm. per cent NaCl and the standard deviation of 0.0171 gm. indicates that approximately two-thirds of the observations falls within the range 0.8858 to 0.9200 gm. per cent NaCl. In Table IV the results with the thirty boys and twenty girls are analyzed separately. The probable error of the average for boys is 0.0020, for girls, 0.0026, while the probable error of the difference is 0.0034. Since the difference between the vapor pressure for boys and girls is 0.0047 gm. per cent NaCl, the values here obtained fail to indicate a significant difference between the vapor pressure of blood for boys and girls. This finding is at variance with that of Margaria (1), who has shown a significant difference in vapor pressure between men and women, the value for women being slightly lower than that of men. As may be seen in Table IV, the values of adults are higher than those of children, those for men being roughly 4.5 per cent greater than the measurements on boys, while the values for women are about 2.5 per cent higher than those of girls. Furthermore, a comparison of the adult and child groups by means of the standard deviations or by computing the coefficients of variation suggests that a wider dispersion of values among the latter may be expected.

#### SUMMARY

1. The technical details of construction, operation, and calibration of a thermopile devised by Hill are given.

2. The values of the vapor pressure of infants' and children's defibrinated whole blood in terms of an osmotically equivalent solution of sodium chloride are presented. The average value for 50 samples of blood was 0.9029 gm. of NaCl per 100 gm. of H<sub>2</sub>O with a probable error of the average of 0.0016.

3. The data so obtained are treated statistically. The probable errors of measurement for one, two, three, and four determinations per sample are tabulated.

The statistical treatment of the data was carried out by Dr. A. A. Weech to whom I wish to express my deep appreciation for his very kind assistance.

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## CARNOSINE AND ANSERINE IN MAMMALIAN SKELETAL MUSCLE\*

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Two imidazole derivatives, carnosine and anserine, have been isolated from vertebrate skeletal muscle. Carnosine may be readily obtained from the ox (1), horse (2), pig (3), etc. Anserine, a methyl carnosine, has been isolated from birds, a reptile (4), fish (5), and certain mammals (6). The presence of the two compounds in muscles from the same animal has been adequately demonstrated only once: Hoppe-Seyler, Linneweh, and Linneweh (4) obtained anserine and a small amount of carnosine from the crocodile. We report below information which, together with the data in a previous paper (6), demonstrates that both carnosine and anserine are present in the muscles of several mammals.

*Isolation of Anserine*—The procedure of Ackermann, Timpe, and Poller (7) as modified by us (6) was employed for anserine isolations. Further modifications in the original procedure were made by precipitating copper anserine from aqueous solution with acetone and recrystallizing from ammonia, steps used by von Fürth and Hryntschak (8) and Kuen (9) in the estimation of carnosine. In outline, the present isolations were made as follows. A concentrated protein-free aqueous extract of muscle was precipitated with mercuric sulfate and alcohol. The mercury precipitate was decomposed with hydrogen sulfide and the solution was

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then fractionated with barium hydroxide and silver nitrate. The lysine fraction was freed from silver and barium, concentrated, and precipitated with mercuric sulfate and alcohol. The mercury precipitate was decomposed, and the resulting solution, freed from sulfate, was concentrated and treated with copper carbonate. The copper anserine was precipitated by adding 5 volumes of acetone and allowing the mixture to stand 24 to 72 hours at 0°. The copper salt was dissolved in a minimum quantity of ammonium hydroxide (sp. gr. 0.90) and centrifuged. The ammoniacal solution was decanted from the insoluble residue, and diluted with 4 or 5 volumes of water. The ammonia was allowed to evaporate spontaneously, the last traces being removed *in vacuo* over sulfuric acid. Any decrease in volume of liquid was made up by adding distilled water. When crystallization appeared complete, usually in 24 to 36 hours, the crystals were collected in a centrifuge tube and washed three times with small quantities of distilled water. The copper salt was recrystallized two or three times from ammonium hydroxide and finally dried *in vacuo* over sulfuric acid. In one preparation (opossum) leaching out the copper anserine with a little dilute ammonium hydroxide reduced the color value from 2.74 to 1.29 per cent.

*Isolation of Carnosine*—Several difficulties arose in the isolation of relatively pure carnosine from muscles low in the compound. In dog muscle the carnosine determined colorimetrically accounted for only 2 or 3 per cent of the total extractive nitrogen. In the first carnosine fraction obtained with silver nitrate and barium hydroxide, the carnosine (diazo, colorimetric) nitrogen amounted to 20 per cent of the nitrogen present. Two more fractionations with silver and baryta raised the carnosine nitrogen to 60 per cent of that present in the silver precipitate. Owing to losses of carnosine, only two or three treatments with silver and baryta were practicable.

The precipitability of carnosine changed considerably as the isolation proceeded. From a relatively crude extract most of the carnosine precipitated with silver and baryta in the pH range 8 to 11. In the second and third fractionations it precipitated almost completely between pH 6 and 7.5. In the later stages of the preparation it was precipitated by mercuric sulfate much more

readily than in the early stages. In the final precipitation with mercuric sulfate most of the carnosine came down from aqueous solution if the pH were adjusted to 4.0 to 4.5 with barium hydroxide.

The isolation of carnosine from dog and cat muscle was further complicated by the fact that the fractions had been stored for months in the ice box before they were studied. Under such conditions it is our experience that the difficulties of isolation are greatly increased and the yields are low. With these two preparations, attempts to prepare the copper salt resulted in a green amorphous wax. In these instances the material was dissolved in approximately 2 M sulfuric acid and the copper removed with hydrogen sulfide. The resulting solution, freed from sulfide, was treated with about 200 mg. of mercuric sulfate per gm. of carnosine present in 500 cc. volume. After standing overnight, the brownish precipitate was removed by centrifuging. The supernatant fluid, which contained 80 or 90 per cent of the carnosine, was treated with mercuric sulfate in excess and enough barium hydroxide to keep the pH at 4 or 5. This precipitate, containing most of the carnosine, was decomposed, the sulfate removed quantitatively with barium hydroxide, and the carnosine converted into the copper salt. A crop of clean blue hexagons resulted in both preparations.

Crude preparations of copper anserine and copper carnosine contain too little nitrogen and show low decomposition points, so that correct values for each constitute good evidence of freedom from unknown impurities. Carnosine may be freed from anserine with ease from solutions not too concentrated, because carnosine precipitates with silver and baryta, while anserine does not. It is far more difficult to remove small amounts of carnosine from anserine. The color values (6) of copper anserine indicate the extent of admixture of copper carnosine. That the copper carnosine preparations were relatively free from copper anserine was indicated by the appearance of the crystals and by the color values. The color values for the preparations of copper carnosine mentioned in this paper varied from 100 to 108 per cent of the expected values calculated from our standard carnosine solutions. The reason for the high values is uncertain.



## EXPERIMENTAL

*Dog. Copper Carnosine*<sup>1</sup>—Typical blue hexagons were obtained which decomposed at 215–216°.<sup>2</sup> The preparation was recrystallized<sup>3</sup> three times from ammonia and dried at 120° for analysis.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated.	N 18.36
	Found.	" 17.97 (Kjeldahl)

*Cat. Copper Carnosine*<sup>1</sup>—The typical blue hexagons decomposed at 218–219°.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated.	N 18.36
	Found.	" 18.36 (Kjeldahl)

*Deer (Odocoileus virginianus)*—5.06 kilos of clean skeletal muscle were obtained mainly from the chest and forelegs of two animals. The tissue was kept at 5–10° for 48 hours prior to extraction.

*Copper Anserine*—4.39 gm. of typical lilac-red crystals which decomposed at 220° and gave a color value (6) of 0.6 per cent (carnosine) were isolated.

$C_{10}H_{16}N_4O_3 \cdot CuO$ .	Calculated.	N 17.53
	Found.	" 17.32 (Dumas)

*Copper Carnosine*—0.92 gm. of typical blue hexagons which decomposed at 219° was isolated.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated.	N 18.36
	Found.	" 18.58 (Kjeldahl)

*Opossum (Didelphis virginiana)*—Seven animals, killed in the laboratory, were dissected and the skeletal musculature was worked up immediately. The weight of muscle was 5.22 kilos.

<sup>1</sup> This was obtained from silver precipitates of the extract which yielded the anserine described in a previous paper (6).

<sup>2</sup> All decomposition temperatures have been corrected. The decomposition points are of value in showing the absence of certain impurities present in crude preparations. However, the decomposition points are unsatisfactory for demonstrating the presence of copper anserine in copper carnosine preparations and *vice versa*.

<sup>3</sup> The recrystallization of copper carnosine, by dissolving it in strong ammonia and diluting after removal of some insoluble foreign material, is not ideal. The analyses of the preparations improved but the decomposition points became less satisfactory.

*Copper Anserine*—2.25 gm. of lilac-red crystals which decomposed at 229° and gave a color value of 1.29 per cent were isolated.

$C_{10}H_{16}N_4O_3 \cdot CuO$ .	Calculated.	N 17.53
	Found.	" 17.52 (Dumas)

*Copper Carnosine*—0.33 gm. of typical blue hexagons which decomposed at 220–221° was isolated.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated.	N 18.36
	Found.	" 17.75 (Dumas)

*Gnu (Connochætes taurinus)*—A healthy animal, injured in an accident, was killed by its keeper and the carcass kept in an ice box 16 hours prior to dissection of 8 kilos of muscle from the hind legs. The diazo method applied to this tissue indicated 0.017 per cent carnosine, the lowest value which we have obtained on mixed skeletal muscle from any mammal. It is rather striking that an animal closely related to the ox should show only a trace of carnosine and large amounts of anserine.

*Copper Anserine*—9.95 gm. of typical lilac-red crystals which decomposed at 225–227° and gave a color value of 0.31 per cent were isolated.

$C_{10}H_{16}N_4O_3 \cdot CuO$ .	Calculated.	N 17.53
	Found.	" 17.37 (Dumas)

*Copper Carnosine*—33 mg. of typical blue hexagons which decomposed at 216–220° were isolated.

$C_9H_{14}N_4O_3 \cdot CuO$ .	Calculated.	N 18.36
	Found.	" 18.0 (Dumas)

*Llama (Lama glama)*—A healthy animal was killed and the muscles dissected from the hind legs immediately. The total weight of muscle used was 6.8 kilos.

*Copper Anserine*<sup>4</sup>—4.94 gm. of typical lilac-red crystals which decomposed at 225° and gave a color value of 2.7 per cent were isolated.

$C_{10}H_{16}N_4O_3 \cdot CuO$ .	Calculated.	N 17.53
	Found.	" 17.70 (Dumas)

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<sup>4</sup> This isolation was carried out by Mr. John Zapp.

## DISCUSSION

We have found both carnosine and anserine in the skeletal muscles of five mammals including two carnivores, a marsupial, and two ruminant herbivores, whereas carnosine alone has been found in the herbivorous horse and ox. Of these two compounds, anserine was certainly present in excess in the muscles of the dog and gnu. This is shown in Table I by a comparison of the compositions of the muscles, the calculations being based on (1) the recoveries of anserine, which yield data naturally much too low, and (2) the diazo color determinations for carnosine, which are probably a little too high. With figures based on the yields of

TABLE I  
*Concentrations of Carnosine and Anserine (in Per Cent of Wet Muscle) in Mixed Muscles Calculated from Diazo Colorimetric Determinations and from Isolation of Compounds*

	Carnosine		Anserine Isolation
	Diazo color	Isolation	
Dog.....	0.05	0.003	0.10
Cat.....		0.025	0.09
Deer.....	0.15	0.014	0.066
Gnu.....	0.017	0.0003	0.094
Opossum.....	0.076	0.005	0.033
Llama.....			0.054

carnosine obtained by isolation, the comparison is even more striking, is possibly more nearly correct, and suggests that the mixed muscles from the cat, deer, and opossum may also contain as much (or more) anserine as carnosine.

## SUMMARY

Data presented in this and a previous paper demonstrate that anserine and carnosine are present together in the skeletal muscles of the dog, cat, deer, gnu, and opossum. Anserine has been isolated from the llama.

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## SARSASAPOGENIN. II

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In previous communications<sup>1,2</sup> it was shown that methyl cyclopentanophenanthrene (Diels' hydrocarbon) can be isolated from the products of the dehydrogenation of both sarsasapogenin and gitogenin with selenium. This observation may now be accepted as a fairly certain indication that these sapogenins possess the nuclear skeleton of the sterols and bile acids. Since our earlier work a constantly mounting number of analytical data with sarsasapogenin itself and with a number of its derivatives have convinced us that it is necessary to revise the hitherto accepted formula of Power and Salway<sup>3</sup> ( $C_{26}H_{42}O_3$ ) to  $C_{27}H_{44}O_3$ . Such a formula now fits in well with the most plausible interpretation of the formation on dehydrogenation not only of Diels' hydrocarbon but also of the more volatile product first obtained by Ruzicka and van Veen,<sup>4</sup> a methyl hexyl ketone. The latter as well as the unsaturated ketone,  $C_{28}H_{44}O_3$ , previously described by us and produced by the action of hydrochloric and acetic acids on sarsasapogenin appears definitely to have its origin in the side chain of this genin. Although the exact identities of these ketones have not yet been established, it is possible that they represent the full extent of the side chain of the sapogenin itself, so that the remaining 19 carbon atoms would be just sufficient to complete the tetra-

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<sup>1</sup> Jacobs, W. A., and Simpson, J. C. E., *J. Biol. Chem.*, **105**, 501 (1934).

<sup>2</sup> Jacobs, W. A., and Simpson, J. C. E., *J. Am. Chem. Soc.*, **56**, 1424 (1934).

<sup>3</sup> Power, F. B., and Salway, A. H., *J. Chem. Soc.*, **105**, 201 (1914).

<sup>4</sup> Ruzicka, L., and van Veen, A. E., *Z. physiol. Chem.*, **184**, 69 (1929).

cyclic portion of the sterol structure, including the two methyl groups on carbon atoms (10) and (13).

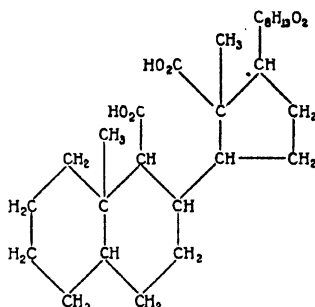
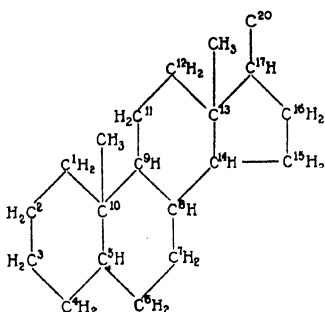
In order to substantiate such indications we have made initial attempts at the oxidative degradation of sarsasapogenin. Although this work is still incomplete, it has furnished, we believe, suggestive data in regard to the structure of this substance. A résumé of this new experience follows.

Oxidation of sarsasapogenin or of its ketone, sarsasapogenone, with alkaline hypobromite produces a mixture of acids of which the main constituent is a crystalline *dibasic acid*. The analysis of this acid has indicated a formula  $C_{27}H_{42}O_6$ . This was substantiated by the study of its *dimethyl ester*. Both the acid and its ester are characterized by their comparative insolubility in the common solvents. The same acid is formed together with a non-crystalline mixture of acid material when the sapogenin is oxidized under gentle conditions with chromic oxide in acetic acid solution, and also when sarsasapogenone is oxidized with Kiliani's chromic acid solution. From the mixture of crude acids in the last two cases a second crystalline acid has been isolated. The analysis of this acid points to a formula  $C_{27}H_{40}O_5$ . Its properties indicate that it is a *monobasic keto acid*, and it forms a *monomethyl ester*. This acid in contradistinction to the dibasic acid is extremely soluble in most solvents but forms a beautifully crystalline sodium salt which is very suitable for its isolation. Although the ester reacts readily with ketone reagents, the products proved to be amorphous.

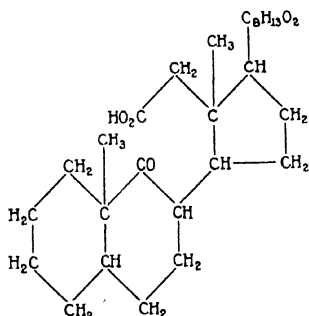
The production of these two acids indicates the presence of the grouping  $---CH_2 \cdot CHOH \cdot CH$  in sarsasapogenin. It can be seen from Formula I that possible positions for the hydroxyl group may be on carbon atoms (4), (6), (7), (11), (15), and (16). Certain data, however, in particular the results of a study of the comparative behavior of the esters of these two acids towards saponification, appear to justify the provisional attachment of the secondary hydroxyl group to  $C_{11}$ , although it has been shown that caution must be exercised on occasion in the interpretation of saponification data.<sup>5</sup>

<sup>5</sup> Ruzicka, L., Waldmann, H., Meier, P. J., and Hösli, H., *Helv. chim. acta*, **16**, 169 (1933).

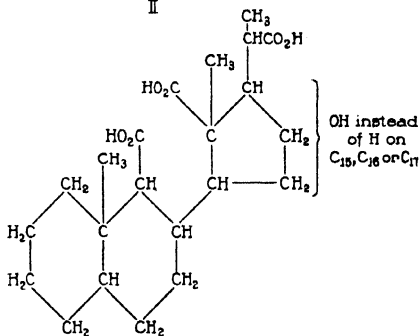
The ester of the keto acid  $C_{27}H_{40}O_5$  is extremely labile and can be completely saponified in 0.05 N alkali. In the case of the ester of the dibasic acid, however, both ester groups are relatively resistant. One of them appears to be but slightly attacked by 0.1 N alkali. Only after 4 hours boiling with 0.5 N alkali did the consumption of alkali approach 1 equivalent. From this mixture a *half ester* was obtained, so that one of the ester groups remained unattacked even under these conditions.



II



III



IV

In the case of the keto acid, therefore, there appears to be present the grouping  $-\text{CH}_2\cdot\text{COOH}$ . In the case of the dibasic acid the most resistant carboxyl group is probably attached to a quaternary carbon atom  $(\text{>C}\cdot\text{COOH})$  and the other carboxyl group to a tertiary carbon atom  $(\text{>CH}\cdot\text{COOH})$ . The only position



for the hydroxyl group in sarsasapogenin which satisfies these requirements is  $C_{11}$ . The formula for the dibasic acid would consequently be as in Formula II and that for the keto acid as in Formula III.

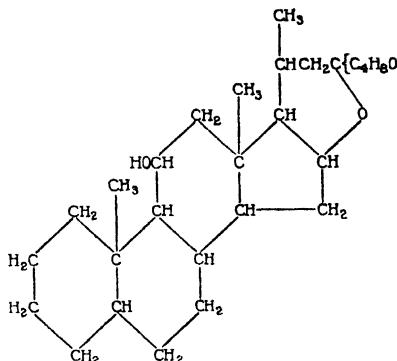
It will be noted that Formula III would require a composition  $C_{27}H_{42}O_6$  for the monobasic keto acid. Such a formula appears, however, to be definitely excluded by the analytical results which dictate a formula  $C_{27}H_{40}O_6$ . The latter formula requires the presence of a double bond in the molecule which must be formed during the oxidation. Although the ester of the acid gives no coloration with tetranitromethane, it can be oxidized with perbenzoic acid to a crystalline *oxide*,  $C_{28}H_{42}O_6$ . On catalytic hydrogenation, the unsaturated ester absorbs approximately 1 mole of hydrogen. However, no crystalline reaction product could be isolated. The resulting amorphous material no longer reacted appreciably with perbenzoic acid, which indicated that hydrogenation of the double bond was the major reaction. Further studies in regard to the double bond are in progress.

When the keto acid is treated with 1 mole of bromine in acetic acid solution a *monobromo keto acid*,  $C_{27}H_{39}O_5Br$ , is formed and can be isolated readily. Bromination of sarsasapogenone under the same conditions gives a mixture which has not as yet been resolved. With 2 moles of bromine the result was also a mixture.

Oxidation of the dibasic acid,  $C_{27}H_{42}O_6$ , with fuming nitric acid yields a *dibasic lactone acid* in moderate yield. Analyses of this acid and of its *dimethyl ester* indicate a formula for it of  $C_{22}H_{32}O_6$ . The lactone group of the acid is readily opened by 0.1 N alkali. In the case of the dimethyl ester, besides the lactone group only one ester group is saponified by this treatment. The second ester group resists even 0.5 N alkali. On reacidification, relactonization at once occurs with the formation of the *monomethyl ester* of the dibasic lactone acid which on reesterification is converted into the original lactone dimethyl ester. The resistance of the second ester group in this substance recalls that of one of the ester groups of the dimethyl ester,  $C_{29}H_{46}O_6$ , and indicates the identity of the two resistant groups in the two substances. The loss of carbon atoms during this oxidation has clearly taken place at the side chain in a manner probably analogous to the oxidation observed by Windaus and Linsert<sup>6</sup> in the case of gitogenic acid. Whether 5

<sup>6</sup> Windaus, A., and Linsert, O., *Z. physiol. Chem.*, **147**, 275 (1925).

carbon atoms of the side chain have been lost in the latter case instead of 4 as postulated by Windaus and Scheckenburger<sup>7</sup> on the basis of their formulation of gitogenin is a question requiring further investigation. In the case of sarsasapogenin the evidence favors the conclusion that 5 and not 4 carbon atoms are removed, and the highly probable identity of the oxygenated side chains of both sarsasapogenin and gitogenin has already been demonstrated by us.<sup>1</sup> The hydroxy tribasic acid produced by saponification of the lactone group of the dibasic lactone acid may possibly have a structure as given in Formula IV, since the ketone  $C_8H_{16}O$  obtained on selenium dehydrogenation of sarsasapogenin is almost certainly a methyl ketone (unpublished work) even though its identity has



not yet been established. From this it would follow that the first 3 carbon atoms of the side chain must have the ordinary sterol configuration. There is as yet no evidence at hand as to which one of the carboxyl groups assumed to be attached to  $C_{20}$  and  $C_9$ , respectively, is involved in the lactone group, the hydroxyl of which is presumably produced by the opening up of one of the original oxidic linkages of the side chain. Lactonization with a carboxyl on  $C_{20}$  would necessitate the attachment of this hydroxyl and therefore of one end of the original oxidic bridge to  $C_{15}$  or  $C_{16}$ . On the other hand, lactonization involving a carboxyl group on  $C_9$  would apparently fix (as the nearest position to this

<sup>7</sup> Windaus, A., and Scheckenburger, A., *Ber. chem. Ges.*, **46**, 2628 (1913).

carboxyl group) the hydroxyl group on C<sub>17</sub>; at best then an  $\epsilon$ -lactone. The first possibility appears on general grounds to be the more likely. Perhaps against this view is the fact that the labile ester group of the lactone dimethyl ester is readily saponified by 0.1 N alkali towards which in the original dimethyl ester, C<sub>29</sub>H<sub>46</sub>O<sub>6</sub>, the less stable ester group is fairly resistant. It is, however, possible that the new lactone grouping in the lactone dimethyl ester can affect the degree of lability of the ester group attached to C<sub>9</sub>. Attempts to confirm this interpretation as well as to degrade sarsasapogenin to a known bile acid derivative are in progress. On the basis of the above interpretation of the data at hand, a provisional structure might be proposed as in Formula V for sarsasapogenin, with necessary reservations and with the exact nature of the side chain still to be determined.

#### EXPERIMENTAL

*Sarsasapogenin*.—This was prepared from Mexican root by the method previously described.<sup>1</sup> With acetone as solvent, a constant melting point of 199.5–200° was obtained which was unchanged on crystallization from alcohol. When recrystallized from ethyl acetate, however, the melting point was lowered somewhat to 194–195° without affecting the analytical figures, due apparently to polymorphism.

$[\alpha]_D^{24} = -76.5^\circ$  ( $c = 2.16$  in chloroform).

$[\alpha]_D^{24} = -54^\circ$  ( $c = 0.66$  in methyl alcohol).

C <sub>26</sub> H <sub>42</sub> O <sub>3</sub> .	Calculated.	C 77.55, H 10.52
C <sub>27</sub> H <sub>44</sub> O <sub>3</sub> .		77.82, 10.65
Found.		77.90, 10.59
		77.92, 10.52
		77.99, 10.70
		77.94, 10.48
		77.88, 10.57

*Oxidation of Sarsasapogenin with Hypobromite.* The Dibasic Acid, C<sub>27</sub>H<sub>42</sub>O<sub>6</sub>.—5 gm. of sarsasapogenin dissolved in 150 cc. of pyridine were shaken for approximately 48 hours at room temperature with an aqueous solution of hypobromite prepared from 200 cc. of 4 per cent NaOH and 13.5 gm. of bromine. The solution was then filtered from unattacked genin (approximately 3 gm.) which was thoroughly washed with water. The filtrate and washings

were left overnight under reduced pressure in order to remove a portion of the pyridine and then filtered from traces of amorphous material. After cooling to  $0^{\circ}$  the filtrate was treated with 100 cc. of 10 per cent sulfuric acid followed by 100 cc. of saturated ammonium sulfate. After short standing the crude acid was collected, thoroughly washed with hot water, and dried. The yield was 1 gm. After recrystallization from acetic acid, the acid formed glistening, flat needles which melted at  $280^{\circ}$  with decomposition. It is very sparingly soluble in the common solvents and separates from ethyl acetate in small rhombs. For analysis samples were crystallized from acetic acid and also from ethyl acetate. These were dried at  $145^{\circ}$  and  $100^{\circ}$  respectively and 15 mm.

$C_{27}H_{42}O_6$ .	Calculated.	C 70.08, H 9.16
$C_{26}H_{40}O_6$ .	"	" 69.59, " 8.99
	Found.	" 70.27, " 9.21
		" 70.04, " 9.20
		" 70.42, " 9.06

12.600 mg. of substance on direct titration against phenolphthalein required 0.541 cc. of 0.1 N NaOH. Calculated for 2 equivalents, 0.546 cc.

12.790 mg. of substance required 0.578 cc. Calculated for 2 equivalents, 0.554 cc.

*Dimethyl Ester*—This was prepared from the acid with diazomethane and crystallizes from chloroform-methyl alcohol in plates which melt at  $216-217^{\circ}$ . The ester is sparingly soluble in ether and the alcohols but readily in chloroform.

$C_{29}H_{46}O_6$ .	Calculated.	C 70.97, H 9.45, $OCH_3$ 12.65
$C_{28}H_{44}O_6$ .		70.54, 9.31, " 13.02
	Found.	70.79, 9.20, " 12.84
		71.28, 9.24
		71.18, 9.31
		71.13, 9.55

17.093 mg. of substance were refluxed in 2 cc. of alcohol and 3 cc. of 0.5 N NaOH for 4 hours and titrated back against phenolphthalein. Calculated for 1 equivalent, 0.070 cc.; found, 0.076 cc.

38.290 mg. of substance consumed 0.130 cc. of 0.5 N NaOH. Calculated for 1 equivalent, 0.156 cc.

0.1 N alkali was found inadequate. 14.330 mg. of substance

were refluxed with 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours. Calculated for 1 equivalent, 0.293 cc.; found, 0.115 cc.

The solution from the second saponification experiment was concentrated, acidified to Congo red with dilute hydrochloric acid, and extracted with ether. The ethereal solution was then repeatedly shaken out with small quantities of 2 per cent sodium hydroxide. The alkaline solution thus obtained was largely diluted to dissolve the sparingly soluble sodium salt, after which it was heated to drive off ether, filtered, and acidified to Congo red. The half ester which was precipitated was collected and found to melt constantly at 224–225° on recrystallization from dilute acetone. It crystallizes in long prismatic needles.

$C_{28}H_{44}O_6$ . Calculated,  $OCH_3$  6.51; found,  $OCH_3$  7.98

*Sarsasapogenone*.—Sarsasapogenin itself has been found to be very readily acetylated on standing in acetic acid solution, so that this has proved on occasion to be a complication during the preparation of the ketone. The following conditions were finally adopted for the preparation of this substance.

A suspension of 7.5 gm. of the genin in 5 cc. of water and 100 cc. of acetic acid was treated at 25–30° during 50 minutes in portions and with constant shaking with a solution of 1.9 gm. of chromic acid in 4 cc. of water and 30 cc. of acetic acid. Solution gradually occurred and after a time the ketone crystallized. After all of the reagent had been added the suspension was warmed to 50–55° for 10 to 15 minutes and then poured into 3 volumes of water and extracted with ether. The extract was washed repeatedly with water, after which acid constituents were removed by shaking with 5 per cent sodium carbonate. The ethereal solution after concentration left a residue which crystallized from acetone. The most favorable yield was 4.8 gm. The substance melts at 223–224°.

$C_{27}H_{42}O_3$ .	Calculated.	C 78.20, H 10.22
$C_{26}H_{40}O_3$ .	"	" 77.94, " 10.07
	Found.	" 78.01, " 9.90
		" 78.28, " 10.15

Frequently the ketone was contaminated with small amounts of a persistent substance containing a higher percentage of oxygen, which caused slight lowering of the melting point and affected the analytical figures.

*Keto Acid*,  $C_{27}H_{40}O_5$ —The alkaline solutions obtained from oxidation experiments carried out under the above conditions, or from those in which conditions were varied as to temperature and concentration, were concentrated under reduced pressure until crystallization occurred. The sodium salts were redissolved by addition of sufficient water, gently warmed, filtered if necessary, and then acidified to Congo red with dilute hydrochloric acid. In many cases the acid mixture was thus obtained in an easily filtrable form and was collected after addition of ammonium sulfate. If much of the dibasic acid,  $C_{27}H_{42}O_6$ , was present in the crude material, it was removed by digestion with hot ethyl acetate which left most of the dibasic acid undissolved. For isolation of the keto acid contained in the solution, advantage was taken of the lability of its ester and the favorable properties of its sodium salt obtained after saponification of the ester. For this purpose the dry crude acid obtained after removal of the solvent was esterified with diazomethane, and the ester mixture was then saponified. For this purpose it was treated with 10 per cent more than the amount of 0.1 N NaOH required on the assumption that all of the ester mixture consisted of the ester  $C_{28}H_{42}O_6$ . An equal volume of 50 per cent methyl alcohol was then added, and the mixture was refluxed for  $1\frac{1}{2}$  hours. The diluted solution was extracted with ether to remove unsaponified material, and the alkaline solution was then concentrated until the sodium salt of the keto acid crystallized as a mass of long, silky needles. This was collected and washed with 5 per cent sodium carbonate in the centrifuge. The acid was obtained from the salt with dilute hydrochloric acid. On recrystallization from isopropyl ether containing a little acetone, it separated as characteristic large, soft plates which melted at  $161-162^\circ$ . The acid is easily soluble in the common solvents except isopropyl ether and petroleum ether, and moderately so in ether. Samples of a number of preparations recrystallized from different solvents gave on analysis consistent figures. The following are representative analyses.

$C_{27}H_{40}O_5$ .	Calculated.	C 72.92, H 9.07
	Found.	" 73.27, " 9.12
		" 73.15, " 8.86
		" 72.89, " 9.01

*Methyl Ester of the Keto Acid*—This was prepared with diazomethane. It is very soluble in the common solvents except petroleum ether and crystallizes from aqueous methyl alcohol as curved plates which melt at 123–124°.

$C_{28}H_{42}O_6$ .	Calculated.	C 73.31, H 9.24, $OCH_3$ 6.77
	Found.	" 73.22, " 9.02, " 6.40
		" 73.64, " 9.36

0.3744 gm. was refluxed with 20 cc. of 0.1 N NaOH and 7 cc. of methyl alcohol for 70 minutes and then titrated against phenolphthalein. Calculated for 1 equivalent, 8.18 cc.; found, 8.21 cc.

*Bromination of the Keto Acid.* The Acid  $C_{27}H_{39}O_6Br$ —0.1 gm. of the above acid dissolved in 2.5 cc. of acetic acid was treated during 5 minutes with 1.3 cc. of a solution of bromine (6 gm.) in acetic acid (200 cc.). As soon as a trace of hydrogen bromide had been formed the bromine was absorbed as quickly as added. The solution was poured into 3 volumes of water and the precipitate was collected with water. After a preliminary crystallization from acetone-ether it was recrystallized twice from 50 per cent acetic acid, from which it formed long, thin microplates. It melted at 197° with decomposition.

$C_{27}H_{39}O_6Br$ .	Calculated.	C 61.91, H 7.55, Br 15.26
$C_{26}H_{37}O_6Br$ .	"	" 61.28, " 7.32, " 15.70
	Found.	" 61.82, " 7.49, " 15.55

*Oxide of the Keto Ester*—0.67 gm. of the methyl ester,  $C_{28}H_{42}O_6$ , was treated with 15 cc. of an approximately 0.15 N solution of perbenzoic acid in chloroform. The mixture was left at 0° for 120 hours. A control of the reagent and solvent was simultaneously run. On titration, the amount of oxygen consumed by the substance was found to be 60.7 per cent of the theoretical amount required by one double bond. In another experiment of only 24 hours duration the amount absorbed was only 37.5 per cent. The chloroform solution of the first experiment was drawn off and washed successively with 5 per cent sodium carbonate and water. The residue left after concentration was partly crystalline. After repeated recrystallization, first from methyl alcohol and finally from aqueous acetone, the substance was obtained as prismatic needles which melted at 191–192°.

$C_{28}H_{42}O_6$ .	Calculated.	C 70.84, H 8.92
$C_{27}H_{40}O_6$ .	"	" 70.39, " 8.76
	Found.	" 70.90, " 8.96

*Dibasic Lactone Acid*,  $C_{22}H_{32}O_6$ —1 gm. of the dibasic acid  $C_{27}H_{42}O_6$  was gradually treated during 10 to 15 minutes with 5 cc. of fuming nitric acid (1.5) at 0°. The clear solution was then slowly warmed to 75–80° during 20 minutes and then maintained at this temperature for 30 minutes. The clear yellow solution was then treated with 2.5 cc. of acetic acid. Water was added to the hot mixture until the solution became turbid. This was followed by rapid crystallization. The acid was collected and washed with very dilute acetic acid. It was recrystallized by solution in hot acetic acid and cautious addition of water. The yield was about 20 per cent. It recrystallizes in small, thick prisms and melts at 295°. It is practically insoluble in the common solvents except the alcohols and acetic acid. For analysis the substance was recrystallized from acetic acid and was dried at 145° and 15 mm.

$C_{22}H_{32}O_6$ .	Calculated.	C 67.30, H 8.22
$C_{23}H_{34}O_6$ .	"	" 67.94, " 8.43
	Found.	" 67.33, " 8.12

12.085 mg. of substance were titrated against phenolphthalein with 0.1 N NaOH. Calculated for 2 equivalents, for  $C_{22}H_{32}O_6$ , 0.616 cc.; found, 0.646 cc.

To the above solution 3 cc. of 0.1 N NaOH were added and the mixture was refluxed for 4 hours and then titrated back. An additional 0.295 cc. was consumed. Calculated for 1 equivalent, 0.308 cc.

48.810 mg. of substance on direct titration consumed 2.566 cc. and after refluxing with an excess of alkali an additional 1.170 cc. Calculated for 1 equivalent, 1.245 cc.

The solution from the second saponification was concentrated and made just acid to Congo red. The collected precipitate had the same melting point as the original lactone. It was converted into the dimethyl ester which from the melting point and mixed melting point proved indistinguishable from the dimethyl ester which follows.

*Dimethyl Ester*—This was prepared from the acid with diazometh-



ane and is characterized by an unusually high power of crystallization. From methyl alcohol it forms long prismatic needles which melt at 171–172°.

$C_{24}H_{36}O_6$ .	Calculated.	C 68.53, H 8.63
$C_{25}H_{38}O_6$ .		" 69.08, " 8.82
	Found.	" 68.69, " 8.24
		" 68.54, " 8.28
		" 68.90, " 8.50
		" 68.66, " 8.30

12.660 mg. of substance were refluxed with 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours and titrated back against phenolphthalein. Calculated for 2 equivalents for  $C_{24}H_{36}O_6$ , 0.633 cc.; found, 0.602 cc.

48.590 mg. of substance consumed 2.322 cc. of 0.1 N NaOH. Calculated for 2 equivalents, 2.430 cc.

37.730 mg. of substance refluxed for 4 hours with 2 cc. of alcohol and 2 cc. of 0.5 N NaOH consumed 0.374 cc. Calculated for 2 equivalents, 0.359 cc.

The solution from the second saponification experiment given above was concentrated and acidified to Congo red with dilute hydrochloric acid. The precipitated half ester after recrystallization from dilute acetone melted at 213–215°.

$C_{23}H_{34}O_6$ .	Calculated.	C 67.94, H 8.43, $OCH_3$ 7.64
	Found.	" 68.15, " 8.52, " 7.66

This half ester on esterification with diazomethane yielded again the original dimethyl ester.

Oxidation of the dimethyl ester,  $C_{29}H_{46}O_6$ , with nitric acid (under the same conditions as those used in the case of the free acid) yielded the same dibasic lactone acid in poor yield.

# CHEMISTRY OF SLASH-PINE (*PINUS CARIBÆA*, MORELET)

## I. FATTY CONSTITUENTS OF THE PHLOEM

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(Received for publication, February 25, 1935)

This paper is the first report on an extensive investigation under way on the chemical mechanisms involved in the formation and possible translocation of oleoresin within the tree. The necessarily voluminous discussion of the theories of phytosynthesis and metabolism involved has been partly covered already (1). The determination of the nature of the constituents in tissues conceived to be actively associated with the formation of oleoresin is required as a preliminary to further work on seasonal variations. It is hoped that the investigation will not only aid in clarifying the processes of oleoresin synthesis, but also the larger problems of metabolism and translocation within the tree.

The slash-pine (*Pinus caribæa*, Morelet) of the southeastern United States is not only an important and typical oleoresin-producing tree, but is available in extreme profusion in pure stands of young saplings that are well suited for investigations in which a differentiation of tissues is desired. This species has therefore been selected for intensive examination.

Microscopical investigations by Gerry (2) have shown that the phloem of slash-pine is extremely rich in labile materials as compared with woody tissue. Devaux and Bargues (3) demonstrated the presence of either oleoresin or oleoresin-yielding material in the phloem and rays of *Pinus pinaster* (maritime pine). Since the rays are closely associated with the resin passages of the xylem, and form, in effect, a physiological link between these and the

\* Maintained at Madison, in cooperation with the University of Wisconsin.

phloem, the latter was selected as the starting point for the investigation.

The question of the existence of oleoresin as such or in combination prior to its appearance in the resin passages of the xylem cannot be considered as settled, since no entirely reliable staining technique exists for distinguishing between fats and oleoresins *in situ*. Furthermore, no oleoresinous constituents have been detected in the petroleum extract of the phloem. This extract consists entirely of fatty and unsaponifiable material. The ether extract following the petroleum extract apparently contains no free oleoresinous constituents, but does yield a very small amount of a complex glycosidic substance which contains combined oleoresinous bodies. This substance will be discussed in a subsequent article. The present paper deals exclusively with the constituents found in the petroleum (60-70°) extract of the phloem.

#### EXPERIMENTAL

The material examined was collected in April, 1934, in the forests near Cogdell, Georgia. At the time of collection the top growth was in the stage of bud elongation and oleoresin production from working trees was entering the period of vigorous activity. Saplings of 2 to 4 inches diameter were felled and topped well below the preceding season's growth in order to avoid chlorophyll-bearing phloem. The cortex was carefully removed and the phloem peeled from the wood in long, narrow strips. These were dried in a shelf drier in a current of air at 45° for about 9 hours to a brittle condition. They were then broken up and packed in tight containers for shipment to Madison. From tree to container, the longest elapsed time was about 14 hours. At the laboratory the dried material was ground in a Wiley mill to approximately 40 to 60 mesh, packed in air-tight containers, and kept at 3° until ready for extraction.

The ground material contained 5.4 per cent water and 5.5 per cent of material extractable in petroleum of boiling range 60-70°. 38 kilos were completely extracted with such petroleum in a continuous percolator and from the extract 2 kilos of crude fat were recovered by evaporation of the solvent under a partial vacuum. The product was slightly greenish brown in color. It had a saponification value of 137.

The crude fat was saponified with alcoholic potassium hydroxide in the usual manner, the alcohol removed under a vacuum, and the soaps dissolved in water (2.5 liters per 500 gm. of fat). These solutions were exhausted with ether in continuous extractors. The ethereal extracts, upon evaporation, yielded yellow, semicrystalline masses (Fraction A) which were examined. The soap solutions were decomposed with a slight excess of sulfuric acid. A layer of ethereal solution of fatty acids floated above a flocculent precipitate on the aqueous layer. This precipitate was removed by centrifuging and examined (Fraction B). The fatty acids (Fraction C) were collected by extraction of the aqueous liquids with ether and evaporation of the solvent.

All crystalline preparations were dried for analysis at 56°, 0.1 mm., for 24 hours over phosphorus pentoxide. Rotations were observed with a Franz Schmidt and Haensch (Berlin) quartz wedge saccharimeter equipped with a Ventzke scale and an electric sodium lamp as light source. The melting points were made with a Thiele apparatus with a thermometer calibrated against a Bureau of Standards thermometer and are not corrected for stem emergence. Carbon and hydrogen were determined by a semimicromethod developed from the original Pregl procedure.

#### *Unsaponifiable Matter (Fraction A)*

This fraction amounted to approximately 200 gm. of a yellow, oily mass containing some crystalline material. By extensive fractional crystallization from alcohol, six fractions of crystals were obtained (total 3 gm.), all of which gave the Liebermann-Burchard sterol reaction and melted at 137–138°. One of these fractions was purified further by the modified (4) digitonin procedure of Windaus (5). The purified sterol was unchanged in melting point after crystallization from alcohol.

*Analysis*— $[\alpha]_D^{25} = -23.4^\circ$  (0.04 gm. in 5 cc. of  $\text{CHCl}_3$ , 2 dm. tube).

$\text{C}_{28}\text{H}_{50}\text{O}$ . Calculated. C 84.06, H 12.08

Found. " 83.53, 83.56, " 12.33, 12.33

The acetate was prepared by heating with acetic anhydride, and crystallizing from alcohol until it showed a constant melting point of 126°.

*Analysis*— $[\alpha]_D^{25} = -29.7^\circ$  (0.05 gm. in 5 cc. of  $\text{CHCl}_3$ , 2 dm. tube).

$\text{C}_{31}\text{H}_{52}\text{O}_2$ .	Calculated.	C 81.22,	H 11.35
	Found.	" 80.98, 81.10,	" 11.62, 11.64

For purposes of comparison, a sterol of the same melting point from the seeds of *Pinus sabiniana* was purified in the same manner and analyzed.

*Analysis*— $[\alpha]_D^{25} = -22.7^\circ$ ; C 83.64, 83.74, H 12.44, 12.22.

All the available sterol (4 gm.) was purified over the digitonide, acetylated, and the acetate crystallized from alcohol. The last fraction (1 gm.) was rejected, and saponification equivalents determined on the first two by the procedure of Sandqvist and Bengtsson (6).

$\text{C}_{31}\text{H}_{52}\text{O}_2$ .

Calculated.		Saponification equivalent 123
Found.	Fraction 1.	" " 122.48, 123.04,
		average 122.76
	" 2.	" " 121.4, 123.4,
		average 122.4

Since it has been shown that this procedure is far more reliable in determination of empirical formulæ for sterols than combustion analysis, the formula  $\text{C}_{29}\text{H}_{50}\text{O}$  is proposed for this sterol. The melting points are those of sitosterol and the rotations are within the range of possible mixtures of its various stereoisomers. Therefore, although the combustion figures actually agree much better with other formulæ, the substance is probably a sitosterol.

*Alcohol*,  $\text{C}_{16}\text{H}_{34}\text{O}$ —From the mother liquors of the sterol fractions about 1 gm. of a waxy body was obtained, which separated in poorly formed leaflets. It was soluble in all ordinary organic solvents. After several crystallizations from a mixture of alcohol and ether the melting point remained constant at  $74-75^\circ$ . The substance did not absorb bromine and gave no sterol reactions.

*Analysis*— $[\alpha]_D^{25} = +9.8^\circ$  (0.447 gm. in 5 cc. of  $\text{CHCl}_3$ , 2 dm. tube).

$\text{C}_{16}\text{H}_{34}\text{O}$ .	Calculated.	C 79.34,	H 14.05,	mol. wt. 242
	Found.	" 78.84, 79.13,	" 14.19, 14.29,	" " 241 (Rast)

The acetate, prepared by boiling with acetic anhydride, melted at  $64-65^\circ$ .

This alcohol reacted with Beckmann's chromic acid mixture, but when warmed with the latter in the amount calculated for the oxidation of a primary or secondary hydroxyl to aldehyde or ketone, only the starting material was recovered in diminished quantity. No carbonyl compounds could be isolated. The substance gave a negative reaction to Denigès' reagent (7) and is therefore probably not a tertiary alcohol. The known alcohol of the above formula, cetyl alcohol, melts at 49° and is optically inactive. This substance is either a secondary alcohol or a primary alcohol with a branched chain containing an asymmetric center. Probability favors the former. Since it is apparently a hitherto unknown substance, the name isocetyl alcohol is suggested.

TABLE I  
*Distillation of Oily Unaponifiable Material*

Fraction No.	Amount	B.p. (0.005 mm. approximate)	$n_D^{25}$	$D_{25}^{25}$	Remarks
	cc.	°C.			
1	5	80-100	1.4591	0.8749	Temperature rose very rapidly
2	50	169-170	1.4525	0.8731	
3	45	170-172	1.4533	0.8750	
4	25	172-173	1.4557	0.8795	On liquid, part was solid Solid Tarry residue
5	15	190-200	1.4695		
6	3	200-210			
7					

The remainder of the unaponifiable material consisted of 225 cc. of a yellow oil. This was distilled under a pressure of approximately 0.005 mm. as shown in Table I.

The solid portion of Fraction 5 was separated by pressing upon porous tile. After several crystallizations from a mixture of alcohol and ether, the melting point was constant at 74-75° and no depression was observed when mixed with the alcohol,  $C_{16}H_{34}O$ , previously described. Fraction 6 consisted of a mixture of this alcohol with the sterol previously described. From a small portion of Fraction 7, the sterol was again isolated over the digitonide. Hence, it appears that Fractions 5 to 7 consisted largely of these two substances. Aside from a small quantity of a lower

boiling substance in Fraction 1, Fractions 2 to 4 evidently consisted almost entirely of a single body or a mixture of closely similar compounds.

These fractions did not give the Liebermann-Burchard sterol reaction, but developed a reddish orange color with the reagent. From Fraction 3, a crystalline bromide was prepared in glacial acetic acid at 0°. After repeated crystallization from dilute methyl alcohol, it appeared as platelets, melting point 123–125°. It was not pure and was unstable, so could not be prepared for analysis. The original oil of Fraction 3, however, was analyzed with the following results.

$[\alpha]_D = -0^\circ$ ; iodine No. 104.5.			
$C_{10}H_{16}O_2$ .	Calculated.	C 77.59,	H 12.07, mol. wt. 464
	Found.	" 77.72, 77.63,	" 12.13, 12.14, " " 466 (Rast)

The above formula and iodine value are satisfied by a cycle and two double bonds. The substance could not be acetylated or benzoylated. A determination of hydroxyl by the method of Zerewitinoff gave no active hydrogen. No colorations were given with fuchsin bisulfite or sodium nitroprusside, nor could any addition reactions be obtained with a number of carbonyl reagents.

Methoxyl determinations by the procedure of Clark (8), with the temperature of the condenser maintained at 4°, gave 6.6 per cent (theory,  $(OCH_3)$  6.65 per cent). Somewhat higher values were obtained with higher condenser temperatures. The Weber-Tollens reaction (9) for methylene oxide groups gave a distinctly positive result although not nearly so pronounced as in the case of aromatic methylene ethers. This substance is evidently a complex ether containing one methoxyl group and other ether linkages of unknown character.

#### *Fraction B. Isolation of a Phytosterolin*

Fraction B, separated by centrifuging from the ethereal acid solutions, was repeatedly washed with hot water and alcohol. It was practically insoluble in alcohol or ether, but was repeatedly crystallized from hot dioxane as rather poorly defined crystals. Two fractions were obtained: Fraction 1, 0.5 gm., m. p. 290°; Fraction 2, 0.4 gm., m. p. 225°, with not very well defined melting points. Neither fraction was entirely free from ash. Both gave the Liebermann-Burchard sterol reaction.

*Analysis of Fraction 1*

$C_{29}H_{50}O_6$ .	Calculated.	C 72.9,	H 10.4
	Found.	" 72.1, 72.3,	" 10.5, 10.7
		(Corrected for ash 0.32, 0.41)	

The analysis is in fair agreement with the formula for a hexoside of the sterol occurring free,  $C_{29}H_{50}O$ . The substance was hydrolyzed with amyl alcoholic HCl according to Power and Salway (10), and a sterol isolated melting at 136–137°.

*Analysis*— $[\alpha]_D^{25} = -24^\circ$ .

$C_{29}H_{50}O$ .	Calculated.	C 84.06,	H 12.08
	Found.	" 83.40, 83.35,	" 12.22, 12.21

The aqueous portion of the hydrolysate reduced Fehling's solution and gave a positive Molisch reaction, but no osazone could be prepared.

Fraction 2, according to analysis, was obviously impure. It was hydrolyzed as before but was refluxed for 1 hour instead of 2. The sterol recovered melted at 136–137°.

*Analysis*— $[\alpha]_D^{25} = -25^\circ$ ; C 83.53, 83.44, H 12.62, 12.61.

In this case, the aqueous hydrolysate yielded a phenylosazone melting at 204°. Evidently both these fractions consisted of a phytosterolin.

*Fraction C. The Fatty Acids*

*Identification of n-Caproic Acid*—The ethereal solution of fatty acids after removal of the sterolin fraction was evaporated and the residue distilled with steam. The acid distillate was extracted with ether and the ethereal solution shaken out with sodium carbonate solution. From this, by acidification and extraction with ether, 0.5 cc. of an oily acid was obtained, which had the odor of one of the lower fatty acids. A Duclaux distillation indicated that the preparation was probably impure. It was converted over the sodium salt to the *p*-bromophenacyl ester (11). After repeated crystallizations from dilute alcohol the ester melted constantly at 69–70°. The corresponding *n*-caproate (from *n*-caproic acid, Eastman) melted at 70–71°, and the mixture of the two at 69–70°. Mixed melting points with the *n*-valerate (m. p., 63°) and isovalerate (m. p., 68°) gave large depressions. The acid was therefore identified as *n*-caproic acid.



The ethereal solution after extraction with sodium carbonate yielded 0.1 cc. of a neutral fragrant oil with the odor of a high aliphatic alcohol.

The non-volatile fatty acids were separated into solid and liquid fractions according to the procedure of Twitchell (12). When the lead salts of the solid acids were precipitated from 3430 cc. of 95 per cent alcohol per 200 gm. of mixed acids at  $+4^{\circ}$ , the precipitated salts carried down a considerable quantity of semisolid salts which could be easily separated from the solid salts by means of ether. This fraction was later found to consist of almost pure oleic acid, while the fraction obtained from the lead salts soluble in alcohol at  $4^{\circ}$  was fairly rich in linoleic.

*Solid Acids*—The solid acids were recovered from the lead salts in the usual manner.

*Identification of Palmitic Acid*—25 gm. of the crude mixture were distilled at 0.01 mm. A few gm. only were distilled over at  $170^{\circ}$ . This product, recrystallized from alcohol, melted at  $62^{\circ}$ . No depression was observed when mixed with palmitic acid (Eastman), melting point  $62.5^{\circ}$ .

*Analysis*

$C_{16}H_{32}O_2$ .	Calculated.	C 75.00, H 12.50
	Found.	" 75.11, " 12.83

The *p*-nitrobenzyl ester melted at  $42-43^{\circ}$  and showed no depression with the corresponding palmitate. The methyl ester gave a saponification number of 208 (methyl palmitate, 208).

*Identification of Dodecosanic Acid (Behenic)*—The main portion of the crude acids was extensively fractionally crystallized from mixtures of alcohol and ethyl acetate without obtaining a pure fraction as determined by melting points and neutralization values. Fractional distillation of the methyl esters gave some pure methyl palmitate, but the higher fractions were mixtures. However, clean separations were obtained by the following procedure.

5 gm. of mixed acids dissolved in 200 cc. of alcohol were neutralized while hot with 0.1 N alcoholic potassium hydroxide. Upon cooling, a potassium salt separated as a gel which was easily broken up by stirring, filtered off by suction, and washed with cold alcohol. Repetition of this process on the acids obtained from the insoluble potassium salts gave clean separations of palmitic acid from the acid contained in the insoluble salt.

The acid recovered from the insoluble potassium salt, and recrystallized three times from a mixture of alcohol and ether, melted constantly at 78–79°.

*Analysis*

$C_{22}H_{44}O_2$ .	Calculated.	C 77.65,	H 12.94,	N. E.* 165
	Found.	" 77.53, 77.74,	" 13.31, 13.13,	" " 164.5

\* Neutralization equivalent.

The *p*-bromophenacyl ester melted at 91–92° and thus cannot be distinguished from the stearate by this means. However, when mixed with the stearate in approximately equal amounts, the melting point dropped to 85°. The methyl ester, prepared by refluxing with absolute methyl alcoholic HCl and recrystallized several times from a mixture of 60:40 isopropyl ether and ethyl acetate, melted constantly at 61–61.5°. However, consistent carbon and hydrogen values could not be obtained upon combustion.<sup>1</sup>

A careful examination of all fractions failed to reveal the presence of stearic acid. The solid acids, therefore, consisted entirely of palmitic and dodecosanic acids, with the latter in slightly larger proportion.

*Liquid Acids. Identification of Oleic and Linoleic Acids*—From the fraction of the lead salts which was only slightly soluble in alcohol at +4° and soluble in ether, about 90 gm. of liquid fatty acids were obtained. These were distilled at 0.01 to 0.015 mm. at 190–194° with very little residue. The distilled acids gave iodine values indicating a mixture and were converted again to lead salts. These were fractionally recovered from alcoholic solution by evaporation. The first two fractions of recovered acids (26 gm., 13 gm.) were mixed and analyzed.

*Analysis*

$C_{18}H_{34}O_2$ .	Calculated.	C 76.60, H 12.06, N. E. 198.6, I No. 90.07
	Found.	" 76.62, " 11.45, " " 195, " " 93 (Wijs)

<sup>1</sup> Some confusion exists as to the melting point of methyl behenate. Values reported are: Klein (*Handbuch der Pflanzenanalyse*, Vienna, **2**, 392 (1932)), 52°; Sudborough, Watson, and Ayyar (*J. Indian Inst. Sc.*, **9A**, 25 (1926)), 52°; Tutin and Clewer (*J. Chem. Soc.*, **105**, 1845 (1914)), 51°; Power and Salway (*J. Chem. Soc.*, **105**, 201 (1914)), 58–59°. Although the preparation reported here is admittedly impure, it melts at 61–61.5°. It is possible that various workers have dealt with isomers.

The *p*-bromophenacyl ester was prepared and found to melt at 46–47°. No depression occurred when it was mixed with the corresponding ester of oleic acid (from Kahlbaum oleic, m. p. of ester, 47°). The main portion of the liquid acids was therefore oleic acid.

That portion of the lead salts which was completely soluble in alcohol at +4° was fractionated by evaporation of the alcoholic solution, and the small fraction most soluble in alcohol separated. The acid recovered from this lead salt had an iodine value of 141. Bromination of this fraction according to the procedure of Farnsteiner (13) yielded a bromide, insoluble in petroleum ether, and melting at 112° (linoleic tetrabromide, m. p. 115°). This bromide was completely soluble in ether, indicating the absence of linolenic acid.

#### Analysis

$C_{18}H_{33}O_2Br_4$ .	Calculated.	C 34.67,	H 5.33,	Br 53.3
	Found.	" 34.65, 34.71,	" 5.58, 5.57,	" 53.1

The liquid acids therefore consisted of oleic and a small proportion of linoleic acids.

#### SUMMARY

A petroleum ether (60–70°) extract of the phloem of *Pinus caribæa*, Morelet, yielded the following substances: a sitosterol,  $C_{29}H_{50}O$ ; a sterolin,  $C_{35}H_{60}O_6$ ; a wax alcohol,  $C_{16}H_{34}O$ ; a complex ether,  $C_{30}H_{56}O_3$ ; and *n*-caproic, palmitic, dodecosanic, oleic, and linoleic acids.

All combustions and rotations and several other determinations on small quantities of material were performed in the Biochemical Research Laboratory of the University of Wisconsin by Dr. Eugene Schoeffel. The services of this laboratory were placed at our disposal by Professor Karl Paul Link.

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## **STUDIES ON KETOSIS\***

### **VI. QUANTITATIVE STUDIES ON $\beta$ OXIDATION**

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The hypothesis that  $\beta$  oxidation is the mechanism whereby fatty acids are oxidized has been generally accepted since the classical experiments of Knoop (1), of Dakin (2), and of Ringer (3). More recently Quick (4) has shown that the total benzoic acid or phenylacetic acid eliminated in combination with glycine and glucuronic acids after feeding the phenyl-substituted fatty acids represents a practically quantitative transformation. The last investigator concludes that the fatty acids are broken down exclusively by  $\beta$  oxidation in the dog. Clutterbuck and Raper (5) have shown that  $\gamma$  and  $\delta$  oxidation occur as well as  $\beta$  oxidation when the longer chain fatty acids are oxidized by hydrogen peroxide. Whether these might be similar pathways of oxidation *in vivo*, however, was not ascertained. In a later paper (6) these investigators failed to observe an excretion of phenylsuccinic acid after the subcutaneous administration to a dog of  $\beta$ -phenylhexoic acid. The former compound should have originated if  $\gamma$  oxidation had taken place.

Although the evidence for  $\beta$  oxidation seems quite convincing as a result of the experimental results reported above, the quantitative transformation of the unsubstituted fatty acids when fed to normal fasting animals should be the final proof to complete it. With the exception of the experiments of Gottschalk (7) who

\* A preliminary report of some of these data has been published (Butts, J. S., *J. Biol. Chem.*, **105**, xv (1934); Butts, J. S., Deuel, H. J., Jr., and Hallman, L., *Proc. Soc. Exp. Biol. and Med.*, in press (1935)).

showed that a marked ketonuria followed the administration of butyric acid to a fasting man, no extensive studies on the oxidation of fatty acids themselves *in vivo* have been made. Shaffer (8) has considered that the fatty acids are oxidized exclusively by  $\beta$  oxidation so that only one diacetic acid residue is formed from each fatty acid chain, irrespective of length.

The procedure of Butts and Deuel (9) for the production of an artificial ketonuria in fasting rats by the administration of regulated doses of sodium acetoacetate by stomach tube twice daily offers a method for comparison of the ketogenic ability of various fatty acids. Although varying amounts of the administered diacetic acid are oxidized in the fasting rats, the average ketone body excretion of a small group of animals after receiving similar amounts of diacetic acid is usually found to be quite close. In the present paper a comparison is made in the ketonuria which develops following the administration of isomolecular quantities of the sodium salts of propionic, butyric,  $\beta$ -hydroxybutyric, diacetic, valeric, caproic, heptic, and caprylic acids.

#### EXPERIMENTAL

Fasting male and female rats of 4 to 6 months of age from our stock colony were used. Most of the animals had previously been on a high carbohydrate diet (Diet II), described elsewhere (10). In some of the later tests the rats had previously received a similar diet to which 5 per cent of desiccated liver had been added (Diet III).<sup>1</sup> Because of the pronounced effect of the inclusion of liver in the diet on the resultant ketonuria (11), separate control tests are reported for the later experiments.

<sup>1</sup> The composition of Diet III is as follows:

	per cent
Whole yellow corn-meal.....	43.0
"    wheat flour.....	28.0
Dried skim milk powder.....	16.0
Desiccated liver.....	5.0
Ground alfalfa leaves.....	4.0
Cod liver oil.....	2.0
Irradiated yeast.....	1.0
CaCO <sub>3</sub> .....	0.5
NaCl.....	1.0

The ground liver which was obtained locally was from beef imported from Argentina. According to Dr. S. Lepkovsky, its vitamin G content was approximately the same as in comparable amounts of fresh liver.

The salts of the acids were administered in isomolecular quantities of 0.259 mole, equivalent to 15 gm. of acetone per sq.m. of body surface, by stomach tube in two divided doses daily. The surface area was computed by the formula of Lee (12) from the body weight at the start of the fast at 5 p.m. prior to the first feeding at 9 a.m. the following morning.

The butyric, valeric, caproic, heptoic, and caprylic acids<sup>2</sup> were chemically pure materials obtained from the Eastman Kodak Company. Their purity was verified by titration with standard base. The lowest values were obtained with butyric acid for which 97.9 per cent was found. The other compounds varied from 98.5 to 100 per cent. Eastman technical propionic acid was used and its strength found to be 95 per cent.

The diacetic acid was obtained by saponification of the ethyl ester with sodium hydroxide, as described elsewhere (13). The sodium salt of  $\beta$ -hydroxybutyric acid was synthesized by the method of Wislicenus (14) as modified by Dunn.<sup>3</sup> The elementary analysis as found by Dr. Dunn was as follows:

Calculated.	C 38.04, H 5.60
Found.	" 37.96, " 5.47

Its identity was also established by the proof of conversion into  $\alpha$ -crotonic acid on distillation with concentrated sulfuric acid. The assay of the sample was carried out by the usual method of Van Slyke (15), considering that only 75 per cent of the  $\beta$ -hydroxybutyric acid is capable of transformation to acetone with the formation of the mercury-acetone compound.

The original sample of heptoic acid as obtained from the Eastman Kodak Company was decidedly toxic and all of the six animals to which it was administered died within a few hours. After the material had been repurified by a refractionation,<sup>4</sup> its toxicity was lost.

In the control experiments in which sodium bicarbonate was

<sup>2</sup> Samples of specially purified caprylic and lauric acids were kindly furnished us by Dr. S. Lepkovsky of the University of California. The Eastman products gave identical results with them.

<sup>3</sup> Dr. M. S. Dunn of the University of California at Los Angeles supplied us with the first sample of this salt. Miss Margaret Gulick in our laboratory subsequently prepared the second sample which was also used in the tests. We wish to express our thanks for both of these preparations.

<sup>4</sup> This purification was carried out by Dr. M. S. Dunn.



administered, the volume of solution containing the appropriate dose of the solute was so large that it was necessary to give it to the rats in three divided doses daily.

The determination of diacetic acid,  $\beta$ -hydroxybutyric acid, and total acetone bodies in the urine was made by the method of Van Slyke (15). The estimation of urine nitrogen was carried out by the Kjeldahl procedure. Tests for urine sugar and albumin were made routinely and the results on the few animals which showed positive tests were discarded.

Since the Van Slyke reagent reacted with valeric acid, the determination of acetone bodies was also made by the Hubbard method (16) in some cases. The latter procedure was found to give negative results with valeric acid. All the other acids when mixed with water in the maximum amounts ingested by any of the rats (about 0.5 gm.) gave blank results when carried through the copper hydroxide precipitation, after which the filtrate was boiled for  $1\frac{1}{2}$  hours with mercuric sulfate solution to which potassium dichromate was added.

### *Results*

A summary of the experiments with diacetic,  $\beta$ -hydroxybutyric, butyric, and caproic acids is given in Table I.

The acetone body excretion calculated on the basis of body surface is practically identical in the tests on  $\beta$ -hydroxybutyric, butyric, and caproic acids with that of the diacetic acid controls. Although the level of the excretion of acetone bodies in the males is somewhat lower than the average value reported elsewhere (17) for a larger group (in which these values are included), it is the mean for rats which were litter mates of the animals which were receiving the other acids with similar diets, and with which the experiments were made simultaneously. The sex difference in susceptibility to ketonuria (9) is shown not only after the administration of diacetic acid but also after butyric and caproic acids were fed.

The proportion of the total acetone bodies which was composed of diacetic acid is practically identical in the butyric and diacetic acid experiments with the females. When caproic acid was administered to female and to male rats, the proportion is somewhat less than after butyric or diacetic acid was taken. In the tests with males the ratio of diacetic acid was considerably higher than with

TABLE I  
*Acetone Body Excretion in Fasting Rats Fed Sodium Salts of Various Fatty Acids in Doses Equivalent to 15 Gm.  
 (As Acetone) per Sq. M.*

Acid in experimental diet	Average weight	Acetone bodies excreted, gm. per sq. m. per day					Per cent diacetic acid in total acetone bodies				
		1st day	2nd day	3rd day	4th day	5th day	1st day	2nd day	3rd day	4th day	5th day
Females.	gm.										
	179	3.51(6)	4.13(5)	6.84(5)	8.03(6)	8.66(6)	33.7	35.7	30.8	28.4	27.6
	176	3.04(7)	4.16(7)	5.30(7)	6.88(6)	7.69(6)	32.4	32.8	30.2	26.1	27.6
	176	3.50(8)	4.06(8)	5.82(7)	6.50(5)	7.89(4)	29.9	23.9	24.2	26.4	25.7
	175	3.74(5)	3.70(4)	8.31(2)	9.36(1)	10.83(1)	7.7	15.4	16.0	17.6	16.0
Males.	178	10.48(2)	9.70(2)				12.6	12.7			
	193	2.45(4)	2.46(4)	3.27(4)	2.91(3)	3.55(3)	51.4	47.6	38.8	42.9	35.2
	201	1.98(3)	2.34(3)	3.40(3)	3.04(3)	3.48(2)	27.3	27.4	30.3	33.2	23.0
	198	3.34(6)	3.14(6)	3.86(6)	3.99(6)	4.54(6)	23.7	27.1	24.3	28.8	32.9

The figures in parentheses represent the number of animals of which this is the average.

\* 1.5 mg. per sq. cm. of body surface.

† 2.5 mg. per sq. cm. of body surface.

females also fed diacetic acid, as well as greater than that of the males which had received butyric or caproic acid. The diacetic acid made up an unusually small amount of the total acetone bodies after *dl*- $\beta$ -hydroxybutyric acid had been fed. This reached a maximum of 17.6 per cent of the total, compared with a minimum of 27.6 per cent on another day after the feeding of diacetic acid. In a few experiments in which larger amounts of  $\beta$ -hydroxybutyric acid were fed (Group II, Table I), the proportion of diacetic acid was not appreciably augmented in spite of a large increase in the excretion of total acetone bodies.

*Experiments with Caprylic Acid*—The effect of caprylic acid on the ketonuria of fasting female rats as compared with diacetic acid is reported in Table II. Because of the divergency in results from those generally accepted, the results of the individual tests are given in detail. The Eastman preparation of caprylic acid was used in the first tests, while that kindly supplied to us by Dr. Lepkovsky was employed in the later ones.

The level of the acetone body excretion in the urine is about twice that in the animals which received caprylic acid as compared with that of the rats to which an isomolecular amount of diacetic acid was fed. The results recorded in the first part of Table II become more pronounced as the experiment is continued and reach their highest value on the 5th day, when the amount of the acetone bodies excreted averages 2.07 times as much in the rats receiving caprylic acid as in those fed diacetic acid. In the tests recorded in the second part of Table II on another group of female rats, which lasted only 2 days, the ratios of acetone body excretion following the two acids are 2.50 and 1.95 respectively. In a third group of animals not recorded here, the acetone body output in the animals given caprylic acid was more than double (2.58) on the 1st day that for a similar number of diacetic acid controls. On the 2nd and 3rd days the ratios were 1.24 and 1.21 respectively. Because of the divergency in our results from those expected after the administration of caprylic acid, the control values on nitrogen excretion are summarized in Table III.

No significant differences in nitrogen excretion sufficient to explain the variations in the level of ketonuria after feeding caprylic and diacetic acids are evident.

*Experiments with Valeric Acid*—Since it was noted that valeric

TABLE II  
*Acetone Body Excretion in Urine of Fasting Female Rats Receiving Sodium Acetoacetate or Sodium Caprylate in Doses Equivalent to 15 Gm. (As Acetone) per Sq. M.*

Rat No.	Body weight	Sodium caprylate (Group I)										Rat No.	Body weight	Acetone bodies, gm. per sq. m. per day			Per cent diacetic acid in total acetone bodies			Acetone bodies, gm. per sq. m. per day			Per cent diacetic acid in total acetone bodies																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
		Acetone bodies, gm. per sq. m. per day					Per cent diacetic acid in total acetone bodies							Acetone bodies, gm. per sq. m. per day			Per cent diacetic acid in total acetone bodies			Acetone bodies, gm. per sq. m. per day			Per cent diacetic acid in total acetone bodies																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
		1st day	2nd day	3rd day	4th day	5th day	1st day	2nd day	3rd day	4th day	5th day			1st day	2nd day	3rd day	1st day	2nd day	3rd day	1st day	2nd day	3rd day	1st day	2nd day	3rd day																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
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acid itself gave a precipitate with Denigès' reagent, which was largely increased when the oxidation with dichromate was employed, the determination of acetone bodies by the Hubbard method was employed also in some of the tests after the administration of valeric acid. Since practically blank values were given

TABLE III

*Average Nitrogen Excretion in Urine of Fasting Female Rats after Sodium Caprylate and Sodium Acetoacetate*

	Substance administered	Urine N, gm. per sq. m. per day				
		1st day	2nd day	3rd day	4th day	5th day
Group I (Table II)	Sodium caprylate	4.50(6)	4.24(6)	3.28(5)	2.95(4)	3.00(4)
	“ acetoacetate	3.89(6)	3.91(6)	3.87(6)	3.69(6)	3.90(6)
Group II (Table II)	Sodium caprylate	5.57(5)	4.85(3)			
	“ acetoacetate	4.60(5)	4.42(5)	4.10(3)		

The figures in parentheses represent the number of animals.

TABLE IV

*Comparative Analysis of Diacetic and Valeric Acids by Van Slyke and Hubbard Procedures*

Acid	Van Slyke procedure							Hubbard procedure		
	Sample taken	Recovery						Sample taken	Total acetone bodies	
		Diacetic acid fraction		$\beta$ -Hydroxy-butyric fraction		Total acetone bodies				
	mg. as acetone	mg.	per cent	mg.	per cent	mg.	per cent	mg. as acetone	mg.	per cent
Valeric.....	150	2.9	1.9	10.2	6.8	13.1	8.7	37.5	0.5	1.3
Diacetic...							*	2.54	2.46	96.9

\* The amount of diacetic acid recovered by the Van Slyke procedure is considered 100 per cent. Its concentration is calculated from this analysis.

for this acid by the latter procedure, it was possible to prove that acetone bodies in small amount were excreted after the administration of valeric acid rather than unmetabolized valeric acid itself. The values for acetone bodies on valeric acid and on diacetic acid by these two procedures are reported in Table IV.

A summary of the results on the valeric acid tests on female rats as well as of the control tests on diacetic acid is given in Table V. Because of the small excretion of acetone bodies obtained in the experiments with the odd numbered fatty acids, the fractionation of these compounds has not been carried out.

Although our results on recovery in the diacetic acid control tests are somewhat unsatisfactory by the Hubbard procedure, the values of acetone bodies in the valeric acid tests determined by the Van Slyke and Hubbard methods are sufficiently close to indicate that practically all of the material giving a precipitate with Denigès' reagent is composed of acetone bodies.

TABLE V

*Comparison of Excretion of Diacetic Acid in Fasting Female Rats after Diacetic and Valeric Acids As Determined by Van Slyke and Hubbard Methods*

Day.....	Acetone body excretion, mg. per day									
	Valeric acid					Diacetic acid				
	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th
Van Slyke method....	27.6	58.1	56.3	60.7	41.3	131.7	137.8	191.1	232.2	199.4
Hubbard method.....	26.7	54.6	49.3	57.3	37.8	86.1	110.4	138.7	185.8	151.2
Per cent recovery by										
Hubbard method....	96.7	94.0	87.5	95.0	91.5	63.2	80.3	72.6	80.0	76.0
No. of experiments....	8	8	7	6	3	4	4	3	2	2

In Table VI the results of the tests with valeric acid on the female rats reported in Table V are summarized, the results for acetone bodies being expressed in gm. per sq.m. for comparison with those after the other acids. A summary of other tests on male animals (in which only the Van Slyke procedure was employed) is included. The values for urinary nitrogen of the animals receiving sodium valerate, sodium heptoate, and sodium propionate, as well as for the sodium acetoacetate controls, were within the normal range.

In distinction to the results on the even chain fatty acids so far investigated, the acetone body excretion amounts only to about one-fourth of that of the diacetic acid controls after valeric acid is administered.

*Experiments with Heptoic Acid*—Six experiments in which our rats lived 1 day or more were completed with the purified heptoic acid. They are recorded in Table VII.

TABLE VI

*Acetone Body Excretion in Fasting Rats Following Administration of Sodium Valerate or Sodium Acetoacetate Equivalent to 15 Gm. (As Acetone) per Sq. M. per Day*

	Acid administered	Body weight <i>gm.</i>	Acetone bodies, gm. per sq. m. per day				
			1st day	2nd day	3rd day	4th day	5th day
Females*	Valeric	197(8)	0.92(8)	1.90(8)	1.91(7)	2.14(6)	2.04(4)
	Diacetic	185(4)	4.52(4)	4.75(3)	6.73(3)	7.82(2)	6.74(2)
Males†	Valeric	252(5)	1.35(5)	1.21(5)	1.02(4)	1.36(2)	0.98(2)
	Diacetic	244(5)	4.64(5)	5.63(5)	6.03(5)	6.78(4)	4.70(4)

The figures in parentheses represent the number of rats of which this is an average.

\* Previously on Diet II.

† Previously on Diet III.

TABLE VII

*Acetone Body Excretion in Fasting Female Rats Receiving Sodium Heptoate or Sodium Propionate Equivalent to 15 Gm. (As Acetone) per Sq. M. per Day*

Sodium heptoate						Sodium propionate						
Rat No.	Body weight	Acetone bodies, gm. per sq. m. per day				Rat No.	Body weight	Acetone bodies, gm. per sq. m. per day				
		1st day	2nd day	3rd day	4th day			1st day	2nd day	3rd day	4th day	5th day
	gm.						gm.					
2144	212	0.11	0.11	0.28	0.72	2150	152	0.17	0.06	0.03	0.00	0.01
2145	184	0.09	0.30	0.35		2152	166	0.03	0.03	0.00	0.00	0.01
2146	170	0.22	0.48			2153	173	0.06	0.86			
2147	158	0.09				2154	226	0.95	0.12	0.09	0.02	0.03
2148	174	0.10	0.10	0.20		2155	212	0.66	0.07	0.04	0.03	0.00
2149	130	0.21										
Average.....		0.14	0.25	0.28	0.72			0.57	0.23	0.04	0.01	0.01

In order to determine whether the failure of the production of an appreciable acetonuria after heptoic acid might be ascribed to an

inability in metabolizing it after its absorption, tests were made to determine whether sodium heptoate might be excreted unchanged in the urine. When 0.656 gm. of heptoic acid (as the sodium soap) was added to urine, 87.5 per cent was recovered by extraction with ether after acidification with  $\text{H}_2\text{SO}_4$ . When four different urine samples from three animals receiving heptoic acid were acidified and extracted with ether, practically blank results were

TABLE VIII

*Urine Acetone Bodies in Fasting Rats Receiving Sodium Bicarbonate in Doses of 2.17 Gm. per Sq. M. per Day*

Rat No.	Body weight	Females Urine acetone bodies, calculated as gm. acetone per sq. m. per day					Rat No.	Body weight	Males Urine acetone bodies, calculated as gm. acetone per sq. m. per day				
		1st day *	2nd day	3rd day	4th day	5th day			1st day *	2nd day	3rd day	4th day	5th day
	gm.							gm.					
2099	186	3.89	0.06	0.35	1.25	1.02	1556	205	2.49	0.07	0.04	0.00	
2100	170	6.70	0.89	0.38	1.02	0.36	1557	222	2.16	0.12	0.05	0.02	0.00
2101	208	3.30	0.07	0.17	0.86	0.37	1558	270	1.28	0.08	0.04	0.02	
2124	161	4.12	2.22	2.87	1.59	1.88	2102	232	3.28	0.18	0.41	0.41	0.88
2125	154	3.13	1.13	2.55	2.05	2.20	2103	257	1.45	0.05	0.06	0.05	0.04
2126	179	3.23	1.49	3.15	3.68	4.13	2104	277	0.82	0.10	0.09	0.07	0.04
2127	185	3.11	0.28	1.14	0.05	0.07	2105	284	3.23	0.20	1.39	0.82	0.42
2128	183	5.52	2.75	2.34	2.78	2.43	2106	319	1.40	0.10	0.11	0.08	0.04
2129	217	2.73	0.57	2.02	1.73	1.95	2107	295	0.77	0.08	0.06	0.04	0.06
2130	208	2.47	0.60	0.26	0.20	0.07	2120	259	1.95	0.19	0.18	0.30	0.09
							2123	314	1.76	0.50	0.42	0.15	0.08
							2131	230	0.79	0.06	0.06	0.04	0.04
							2132	262	1.99	0.15	0.17	0.10	0.21
Average.....		3.82	1.01	1.52	1.52	1.45			1.80	0.14	0.24	0.16	0.17

\* 1.5 gm. of diacetic acid (as acetone) per sq. m. on this day only.

obtained. The amount which might be so accounted for averaged slightly over 2 per cent.

*Experiments with Propionic Acid*—Although the experiments of Ringer (18) indicate that propionic acid is a sugar former, it seemed advisable to conduct experiments with this acid to control further the tests with the other acids. The results are given in Table VII.

Only a small amount of acetone bodies was excreted on the 1st



and 2nd days when propionic acid was fed. The acetonuria was negligible during the last 3 days when sodium propionate was administered.

*Experiments with Sodium Bicarbonate*—If the odd numbered fatty acids be broken down by  $\beta$  oxidation to propionic acid which is oxidizable or convertible to glucose, it is probable that an alkaline residue of sodium bicarbonate would remain which would tend to produce an alkalosis. The amount of sodium bicarbonate so formed should be the molecular equivalent of the soaps which are fed. In view of the results of Booher and Killian (19) in which alkalosis due to overdosing with bicarbonate or to excessive loss of HCl through vomiting was shown to occur concomitantly with a high ketosis, it seemed necessary to carry out control experiments with bicarbonate feeding.

In the following tests 21.7 gm. of sodium bicarbonate per sq.m. of body area, dissolved in water, were given in three equal divided doses daily. This amount is equivalent to the sodium administered when 15 gm. of sodium acetoacetate (calculated as acetone) or equivalent amounts of the salts of other fatty acids are fed per sq.m. The animals used in these experiments had previously been fed on Diet II until the ketonuria during a period of fasting was negligible. The experiments are recorded in Table VIII.

Similar results were obtained on another series of six male and six female rats fed sodium bicarbonate. The exaggerated ketonuria in the females over that of the males receiving a similar treatment is also evident in the second group.

#### DISCUSSION

The experiments reported here offer the first direct proof of the quantitative conversion of butyric and caproic acids into the acetone bodies, after which they suffer the same fate as an isomolecular quantity of diacetic acid. In a large number of rats almost an identical amount of acetone bodies was eliminated in the urine over a period of 5 days when the sodium salts of these acids were fed.

On the other hand, after the administration of caprylic acid, the *excretion* of acetone bodies was consistently greater and in many instances it averaged about twice the amount that was found in the diacetic acid controls. The excretion of acetone bodies was only

0.25 to 0.60 mole per mole ingested in the control experiments with diacetic acid. Therefore the excretion of 1 mole of acetone bodies per mole of ingested caprylic acid is evidence that some molecules of caprylic acid must have each been split into two 4-carbon acids capable of being transformed into acetone bodies. In one experiment (Rat 2116), the acetone body output was 19.96, 20.36, and 20.31 gm. per sq.m. on the 3rd to 5th days respectively, during which period an amount isomolecular to 15 gm. of acetone was being administered. In this instance, the greater acetone body output can only be explained on the basis of  $\delta$  oxidation. Although these experiments may not be interpreted to prove that a quantitative  $\delta$  oxidation may occur in the metabolism of caprylic acid, they can only be construed as indicating that such a process takes place to a considerable extent.

When valeric or heptonic acid is fed, only small amounts of the acetone bodies are excreted in the urine. This speaks very definitely in support of the  $\beta$  oxidation theory. Had any considerable amount of  $\alpha$  oxidation taken place, the butyric or caproic acid so formed would have been largely excreted as acetone bodies in the urine. The ketonuria which developed in the rats receiving heptonic acid was definitely less than in those receiving sodium bicarbonate, so that the results may be considered as blank ones. The values after valeric acid varied between 0.92 and 2.14 gm. per sq.m. in the female rats, which is somewhat higher than in the sodium bicarbonate controls. In the males the values ranged from 0.98 to 1.36 gm. per sq.m., which is considerably higher than in the bicarbonate controls. If the results on valeric acid are to be construed as indication of its transformation into the acetone bodies, such an  $\alpha$  oxidation can only be of minor importance and would account for an average of less than 25 per cent of the total oxidation.

The fractionation of the acetone bodies showed that the percentage of  $\beta$ -hydroxybutyric acid tended to be from 70 to 75 per cent of the total after butyric, caproic, and caprylic acids, and, in most cases, diacetic acid. There was a tendency for the  $\beta$ -hydroxybutyric acid fraction to be lowest in the diacetic acid tests (particularly in the experiments on males), intermediate in the butyric and caproic acid experiments, and highest after caprylic acid. However, after isomolecular amounts of *dl*- $\beta$ -hydroxybutyric were

administered, the  $\beta$ -hydroxybutyric acid fraction in the urine varied from 85 to 92 per cent. Although these experiments indicate the ability of  $\beta$ -hydroxybutyric acid to be converted into diacetic acid, this change has only taken place to about one-half the extent that occurs with the other even chain fatty acids examined. Even after the dose of sodium  $\beta$ -hydroxybutyrate administered was increased to 25 gm. per sq.m. per day, the amount of diacetic acid averaged only about 12.5 per cent on 2 successive days or about one-half of the 25 to 30 per cent obtained after the other acids. This might be explained on the basis that the naturally occurring levo compound is the only one capable of conversion into diacetic acid, while the dextro isomer is unable to undergo any such transformation. Provided such is the case in the metabolism of any quantity of the sodium salt of the racemic acid, only half of the molecules are capable of diacetic acid formation, with the result that the proportion of this fraction is correspondingly reduced. We now believe that this does not necessarily prove that  $\beta$ -hydroxybutyric acid is not a normal direct intermediate in the formation of the acetone bodies, although we so stated it in our preliminary report.

An equilibrium between *excreted* diacetic and  $\beta$ -hydroxybutyric acids similar to that obtained in the urine of fasting human subjects or in that of fasting rats receiving diacetic acid might follow the ingestion of the levo isomer of  $\beta$ -hydroxybutyric acid.

Although the total acetone bodies in the urine after the  $\beta$ -hydroxybutyric acid approximate the value found for diacetic acid during the first 2 experimental days, the much higher level obtained on the 3rd and 4th days might be traced to the presence of a large amount of non-metabolizable *d*- $\beta$ -hydroxybutyric acid. The solution to this problem can only be arrived at by similar studies on the individual isomers.

A variation in absorption of the different sodium soaps cannot be an explanation of the divergencies reported in this paper. Although no tests were made on the amount of fatty acids present in the feces, there was no indication from the amount or the consistency of the stools that the fatty acids were failing to be absorbed. In most cases no feces or only a minimum of well formed stools was excreted after the 1st fast day during which the soaps were being fed. In tests on lauric acid which are not reported

in detail in the present paper, there was a low ketonuria which was accompanied by the elimination of a large quantity of stools having the consistency and color suggestive of the presence of soaps. This excretion continued for the several days, during which the experiment was carried out with several animals. In most cases the laurate was toxic and the animals survived only about 24 hours. The low ketonuria in this case is probably traceable to its non-absorption.

The practically complete digestibility noted by Langworthy and Holmes (20) and Holmes and Deuel (21) for various animal and vegetable fats in human subjects indicates no difference in absorption for those fats having appreciable quantities of short chain fatty acids (as butter or coconut oil) and for those made up almost exclusively of the longer chain compounds.

The large volume of urine (15 to 25 cc.) which was produced daily is evidence that kidney insufficiency could not be the reason for the variation in ketonuria. Moreover, albumin tests were made routinely to determine the presence of any kidney defect. Lastly, the determination of urinary nitrogen was made as a control of kidney function.

The failure in an appreciable ketonuria after heptoic acid administration cannot be due to the inability of the body to metabolize these acids after their absorption. Such small amounts of ether-soluble material could be extracted from the acidified urine of animals fed on heptoic acid that the conclusion must be reached that the elimination of unchanged material by the kidney is inappreciable.

Although Butts and Deuel (9) found no sex difference in the slight acetone body excretion which occurs in normal fasting rats, the female rats develop a much greater ketonuria after sodium bicarbonate administration than do the male animals. In most of the latter group, the acetone body output remains at a blank value during the 4 days that the sodium bicarbonate was administered. The lower ketonuria in the female rats receiving sodium propionate than in the sodium bicarbonate controls suggests that the sugar formed from propionic acid may be able to counteract the ketosis resulting from the alkalosis.

The fact that practically blank values were obtained on the bicarbonate control animals on the 1st day following the adminis-

tration of diacetic acid indicates that the elimination of the acetone bodies from ingested diacetic acid is quite rapid. It must be completely eliminated not later than 16 hours after its administration.

The sex difference in the ketonuria produced after the administration of diacetic acid to fasting rats is definitely shown in the experiments reported here. Such a variation is evident, not only for the ketonuria developed after diacetic acid, but also for that following the administration of butyric, caproic, and valeric acids.

#### SUMMARY

1. The acetone body excretion in fasting rats after the administration of butyric or caproic acid was practically identical with the excretion found when an isomolecular quantity of diacetic acid was fed. (All acids were fed as sodium salts.)

2. The excretion of acetone bodies was approximately twice as great after the administration of caprylic acid as it was after the administration of an isomolecular amount of diacetic acid. This indicates that  $\delta$  oxidation has taken place.

3. The administration of valeric acid was followed by a small ketonuria which exceeded slightly that of the bicarbonate and propionic acid controls. If  $\alpha$  oxidation occurs in the intermediary metabolism of valeric acid, its importance is small.

4. Heptoic acid produces only a slight ketonuria.

5.  $\beta$ -Hydroxybutyric acid makes up 70 to 75 per cent of the total acetone bodies after butyric, caproic, or caprylic acid. It is slightly lower after diacetic acid and reaches 85 to 95 per cent after  $\beta$ -hydroxybutyric acid is fed.

6. The female fasting rat has been shown to be more susceptible to a ketonuria produced by alkalosis than is the male.

7. The sex difference noted previously when diacetic acid was fed to fasting rats is also apparent when butyric, caproic, and valeric acids are administered.

8. Denigès' reagent has been shown to react with valeric acid.

9. It is concluded that both  $\beta$  and  $\delta$  oxidation may play a prominent part in the intermediary metabolism of the fatty acids.

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# THE EFFECT OF THYROID AND THYROXINE ON THE CONCENTRATION OF CREATINE IN THE HEART, MUSCLE, LIVER, AND TESTES OF THE ALBINO RAT

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Comparatively little is known of the effects of hyperthyroidism on the distribution of creatine in the tissues. Abelin and Spichtin (1) analyzed the liver and muscle of eleven rats that had been fed a thyroid preparation and reported extremely large losses of creatine from both, exceeding 60 per cent in some instances. However, these astounding results are open to serious criticism if for no other reason than that their data for the muscle creatine of the five normal control rats varied from 167.75 to 466 mg. per cent, a range of values which stands in marked contrast to the much more consistent data obtained by Chanutin and Kinard (2) and confirmed by the present writer.

Since the present work was begun, Cowan (3) has reported that the creatine content of the ventricles is lowered in thyroxinized rats. Compared with a normal average of 198 mg. per cent, the animals that had received thyroxine for a period ranging from 7 to 33 days contained in the ventricles an average of 152 mg. per 100 gm., while in other animals after a single dose of 0.5 to 0.9 mg., the creatine content was diminished to an average of 121 mg. per cent. As the animals in the chronic group were killed from 1 to 4 days after the last injection, the factor of possible restorative changes in some of them has evidently not been taken into account.

With the exception of several determinations of phosphocreatine in heart muscle, this report is limited to the changes of the total creatine in the heart, skeletal muscle, testes, and liver, produced by the administration of either thyroid substance or synthetic *dl*-thyroxine (Hoffmann-La Roche).



## EXPERIMENTAL

Mature albino rats were employed, the stock being a pure, inbred Wistar strain, reared under very favorable conditions. The basic diet in this laboratory consists of Purina Chow, a dog food preparation. During pregnancy and lactation this is supplemented with a mixture composed of ground Chow, 2 parts; a dried milk preparation, 1 part; brewers' yeast, 0.1 part; and cod liver oil. Sufficient water is added to form a semisolid mixture. This supplementary ration is also furnished to the young for 1 week before and 2 to 3 weeks after weaning.

The daily dose of thyroid (U.S.P., Lilly) was 250 mg. per 100 gm. of body weight. The thyroxine dose was usually 2 mg. daily, administered subcutaneously. In view of the statement (4) that the vitamin B requirement is increased in hyperthyroidism, the rats receiving thyroid and thyroxine were fed an extra amount of brewers' yeast.

For the determination of total creatinine in the tissues, the method of Rose, Helmer, and Chanutin (5) was used, the results being expressed as creatine. While a clearer conception would be gained if the data for each animal were tabulated, for the sake of economizing space, only a summary of the data is given (Table I).

The survival period of the animals receiving thyroid and thyroxine varied within wide limits. At first it was thought that this might be due to individual differences in resistance, but it later appeared that the temperature of the environment was the important factor involved. On similar doses of thyroid, or thyroxine, rats survived much longer in cool than in warm weather. The tendency to lose weight was also less marked at moderate temperatures; indeed under these conditions several rats were observed to gain weight on doses of thyroxine as high as 2 mg. daily.

*Heart*—There was essentially no difference in the creatine content of the ventricles in male and female rats. The average for the normal control group approximated closely the value (198 mg.) obtained by Cowan (3). In the rats receiving desiccated thyroid the most striking change occurred in the heart, where the creatine content was diminished to an average of 56 per cent of normal. Death frequently overtook the thyrotoxic animals in a sitting posture, suggesting heart failure.

TABLE I

*Effect of Thyroid and Thyroxine on Creatine Content of Rat Tissues*

Groups I and II received 250 mg. of thyroid daily per 100 gm. of body weight; Groups III and IV, 2 mg. of thyroxine daily.

	Body weight		Duration of experiment	Creatine, mg. per 100 gm. tissue				Weight of ventricles	Creatine in ventricles
	Initial	Final		Liver	Testes	Muscle	Heart (ventricles)		
Controls (8 males)									
	gm.	gm.	days					gm.	mg.
Minimum.....	144	185		18	245	470	178.8	0.463	0.867
Maximum.....	185	237		39	347	521	218.1	0.787	1.2
Average.....	166	212		30	298	498	192.1	0.546	1.046
Controls (6 females)									
Minimum.....	146	155		20		480	181.0	0.449	0.688
Maximum.....	158	185		27		518	200.0	0.586	1.0
Average.....	151	176		23		497	194.0	0.502	0.903
Group I (16 males, 9 females; autopsy soon after death)									
Minimum.....	152	140	5	28	297	407	83.4	0.600	0.571
Maximum.....	317	256	25	43	364	511	137.0	1.001	1.128
Average.....	208	177	10.6	35	330	452.5	106.8	0.765	0.800
Group II (6 males, 4 females; alive before autopsy)									
Minimum.....	146	122	8	16	271	414	88.3	0.615	0.633
Maximum.....	215	214	27	35	359	504	130.5	0.994	1.050
Average.....	180	173	19	24	330	452	109.2	0.789	0.827
Group III (3 males, 9 females; autopsy soon after death)									
Minimum.....	148	137	10	15.4	316	370	80.5	0.552	0.580
Maximum.....	362	300	30	42	329	462	110.0	1.010	1.010
Average.....	212	175	18	29.7	321	417	96.95	0.784	0.753
Group IV (1 male, 4 females; alive before autopsy)									
Minimum.....	148	138	18	20	313	372	88	0.643	0.658
Maximum.....	283	234	22	38		478	120.7	1.127	1.020
Average.....	195	181	21	32	313	405	101.6	0.893	0.884

Similar results were obtained with synthetic thyroxine. When several animals in a given group died, some of those that were still living were sacrificed and the tissues removed for analysis. The change in the creatine content of the ventricles was practically the same in the two groups. The hearts of the animals that had died contained on an average 96.95 mg. of creatine per 100 gm., or 50 per cent of normal, while those that were sacrificed had an average creatine content of 101.6 mg., or 53 per cent of the normal.

The hearts of the hyperthyroid animals weighed more than normal. That the increase in the size and weight of the ventricles represented a true hypertrophy is indicated by the fact that the water content was the same as that of normal ventricles (Table II). Further evidence of cardiac hypertrophy was obtained by microscopic study, as will be reported elsewhere.

Fieschi and Gavazzeni (6) observed a decrease in the phosphagen fraction of the heart muscle of thyrotoxic rabbits, this being accompanied, according to them, by an increase in free creatine. After a single injection of relatively large doses of thyroxine to guinea pigs, Mattonet (7) found the phosphocreatine fraction diminished in the myocardium. There was a smaller reduction of the pyrophosphate fraction, while the orthophosphate content increased.

The phosphocreatine P fraction was determined in the hearts of three normal rats by the method of Eggleton and Eggleton (8). These were anesthetized with amytal, the chest was opened quickly, and the heart frozen *in situ* with carbon dioxide snow. The results were 4.7, 5.8, and 6.7 mg., respectively. The hearts of three thyrotoxic animals were analyzed similarly. Two of the rats had been kept for 2 days at 35°, at which temperature the intoxication runs a very rapid course. The phosphagen P in each case was less than 1 mg. per 100 gm. The third rat, kept at room temperature, had received thyroid for 5 days previously. The heart contained 2 to 3 mg. of phosphagen P per 100 gm. On the basis of other observations it may be assumed that these animals would not have survived another day, or at most, 2 days, during which time it is perhaps reasonable to suppose that the phosphocreatine would have continued to diminish in the face of an increased demand due to the acceleration of the heart rate. Because of technical limitations these preliminary results are probably not

- \* absolutely quantitative. Nevertheless, even when considered qualitatively, they indicate that heart failure in thyrotoxicosis may be specifically related to the disappearance of the phospho-

TABLE II

*Data Showing Constancy of Water Content in Hearts of Thyrotoxic Rats and Their Normal Controls, in Association with Marked Cardiac Hypertrophy in the Former*

Rat No.	Initial weight		Duration of experiment	Died (D.) or sacrificed (S.)	Weight of ventricles	(Ventricular weight) + (body weight) $\times 100$ based on		Solids	Water	Creatine in ventricles	
	gm.	gm.				Initial weight	Final weight				
			days		gm.			per cent	per cent	mg. per 100 gm.	
52		236		S.	0.581	2.46	23.69	76.31	189		Normal control
53		220		"	0.553	2.52	23.48	76.52	218		" "
56	180	154	10	"	0.615	3.42	3.99	23.60	76.40	105	Thyroid
58	186	165	16	"	0.762	4.10	4.62	23.66	76.34	121	"
67	186	160	20	D.	0.790	4.25	4.93	23.10	76.90	97	"
68	210	171	10	"	0.753	3.59	4.40	23.70	76.30	104	"
69	232	196	12	"	0.968	4.17	4.94	23.87	76.13	103	"
71	230	197	8	"	0.871	3.79	4.42	24.40	75.60	98	"
72	242	225	11	"	0.957	3.95	4.25	23.59	76.41	117	"
73	247	192	21	"	0.797	3.23	4.15	23.90	76.10	98	"
74	174	160	10	"	0.673	3.86	4.20	23.35	76.65	112	"
49	184	156	23	S.	0.626	3.40	4.01	23.17	76.83	168	Rat allowed to recover; no thyroid last 6 days
70	230	247	21	"	0.794	3.80	23.94	76.06	184		Rat allowed to recover; no thyroid last 9 days. Weight before recovery 209 gm.

creatine reserve. How this is associated with the depletion of the total creatine remains to be determined.

*Muscle*.—Our results do not confirm the observations of Abelin and Spichitin (1). The average reduction in muscle creatine was only 9 per cent in the animals receiving desiccated thyroid. In experiments in which the more acute symptoms resulted, the

concentration frequently remained unchanged, or was even elevated. However, the increase was not as pronounced as that observed during fasting (9). A greater tendency to creatine depletion was noted in rats with the more chronic symptoms, and was somewhat more marked in the rats receiving thyroxine.

Because our results differed so strikingly from those of Abelin and Spichtin (1), the possibility was considered that the thyroid preparation which they employed was more active. Accordingly, several rats were fed Burroughs Wellcome tablets in amounts which in their experiments produced an average decrease of 50 to 64.4 per cent within a few days. No such effects were observed in our experiments. After feeding 2000 mg., the muscle creatine was found to be within normal limits (480, 501, and 475 mg. per cent). Indeed the animals lost very little weight and the heart creatine was only moderately diminished (10 to 20 per cent). In one experiment a rat weighing 180 gm. was fed 7000 mg. of the tablets over a period of 11 days. Three specimens of muscle were found to contain 461, 455, and 463 mg. of creatine per 100 gm., respectively. However, in this animal the creatine content of the ventricles was markedly lowered (125.5 mg. per cent).

*Liver*—The results of Abelin and Spichtin (1) were not confirmed. Contrary to their report, a slight increase in liver creatine was frequently noted in the thyrotoxic animals, especially in the experiments in which the intoxication was acute.

Normal values were obtained in the rats fed Burroughs Wellcome thyroid tablets.

*Testes*—There seem to be no data in the literature concerning the effect of hyperthyroidism on the creatine content of the testes. In the rat the testicle is second only to skeletal muscle as regards creatine concentration. Indeed, on the basis of dry weights, the testicle was found to contain more creatine than muscle, 2.2 per cent for the former and 1.99 per cent for the latter. The calculations were based on the results of a series of determinations in which the water content of the testes was found to average 86.5 per cent and that of muscle approximately 75 per cent.

In the hyperthyroid rats the creatine concentration of the testes tended to rise above that for the normal controls, as shown by the data in Table I. The water content remained unchanged. Of particular interest was the observation that while the body weight

of twenty-two male thyrotoxic rats diminished by an average of 12.8 per cent, the testicles apparently retained their initial weights, as may be judged by comparing the ratio, weight per testicle (1.113 gm.) to initial body weight (204 gm.)  $\times 100$  for these animals (0.546), with the data obtained in the series of normal male controls (average weight 212 gm., ratio 0.556) and the value (0.540) calculated from Donaldson's reference tables (10).

The rôle of creatine in the testicle and the factors which tend to increase it in hyperthyroidism remain to be determined. Estimations of the phosphagen fraction indicate that only a small proportion, about 3 to 4 per cent, is present as phosphocreatine.

#### SUMMARY

The administration of thyroid or thyroxine to rats resulted in a marked reduction of the creatine content of the heart, frequently to 50 per cent of normal. Even more striking was the depletion of phosphocreatine observed in a small series of preliminary experiments. Not infrequently death occurred under conditions suggesting heart failure. These results indicate that the disappearance of phosphocreatine from the myocardium may be specifically related to heart failure in acute thyrotoxicosis.

All the hyperthyroid rats showed some degree of cardiac hypertrophy; in some the ventricular weight to body weight ratio was approximately doubled. Because of the hypertrophy, the total creatine per heart diminished only about 20 per cent, on an average.

The change in muscle creatine varied with the duration of the intoxication. In rats with acute symptoms there was usually little, or no reduction, while in cases of more chronic intoxication the concentration was lowered as much as 30 per cent.

The concentration of creatine in the testes of the hyperthyroid rats was moderately increased. In the liver, it varied approximately within normal limits.

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## THE RING STRUCTURE OF THYMIDINE

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The 2-desoxy-ribose nucleosides, first isolated by Levene and London<sup>1</sup> by the hydrolysis of thymus nucleic acid, are characterized by their extreme ease of hydrolysis by very dilute mineral acids. Thus, guanine-2-desoxy-riboside is completely hydrolyzed by heating with 0.01 N hydrochloric acid during 5 minutes. Since the rate of hydrolysis is of the same order as that of the furanosides, it was considered possible that the 2-desoxy-ribonucleosides, similarly to the ribonucleosides, have the furanoside ring structure.

When, however, it was discovered<sup>2</sup> that methyl-2-desoxy-glucopyranoside is almost as readily hydrolyzed, it appeared just as feasible that the natural derivatives might have the pyranoside ring structure. Hence the question of their ring structure was in need of further special investigation.

We have now studied the ring structure of the sugar portion of thymidine (thymine-2-desoxy-riboside). This substance is found to react with triphenylmethyl chloride in pyridine to give a monotrityl derivative. Since it was shown by us<sup>3</sup> that the analogous monotrityl uridine is the 5-substituted derivative, the formation of a monotrityl thymidine might, of itself, be considered a good indication that thymidine is, likewise, a furanoside.

Definite proof of this conclusion was provided by substituting the remaining free hydroxyl group by a tosyl group and examining the behavior of the resulting monotosyl trityl thymidine on treat-

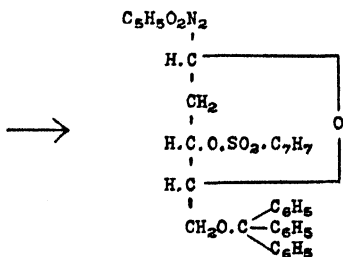
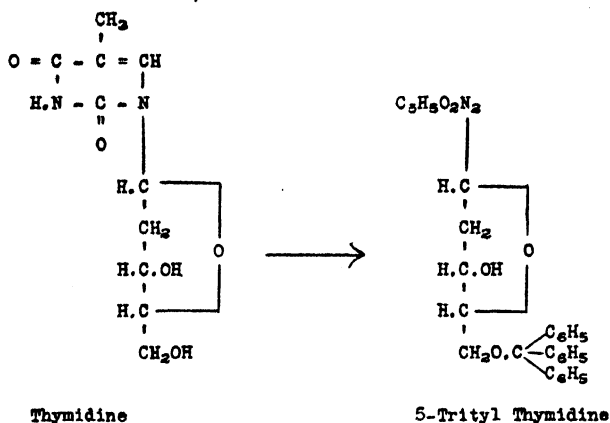
<sup>1</sup> Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929); **83**, 793 (1929).

<sup>2</sup> Bergmann, M., and Breuers, W., *Ann. Chem.*, **470**, 51 (1929). Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **88**, 791 (1930).

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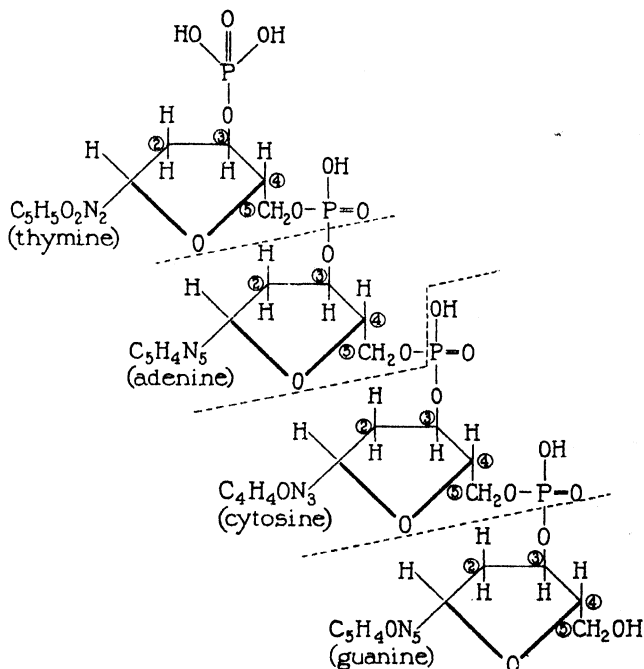
ment with sodium iodide dissolved in acetone. It was found that the tosyloxy group in this case was much more stable than if attached at a primary hydroxyl but, presumably owing to the presence of the  $\text{CH}_2$  group in position (2), the tosyloxy group in position (3) was somewhat less stable than the same group at-



**3-Tosyl 5-Trityl Thymidine**

tached at a secondary hydroxyl group in true sugars. In confirmation, it was found that the secondary tosyloxy group in monotosyl 5-iodothymidine had about the same stability towards the above reagent as had the secondary tosyloxy group in monotosyl trityl thymidine.

Thus it is evident that in *desoxy-ribose nucleic acid* the positions of the phosphoric acid radicles are carbon atoms (3) and (5) of the desoxy-ribose,<sup>4</sup> as in (I). This fact offers the hitherto missing explanation for the differences in behavior of the nucleic acids of the two types. The differences are the following. The ribose nucleic acid is less resistant towards the action of dilute alkali and



Desoxy-ribose nucleic acid (C<sub>35</sub>H<sub>51</sub>O<sub>23</sub>N<sub>13</sub>P<sub>4</sub>)

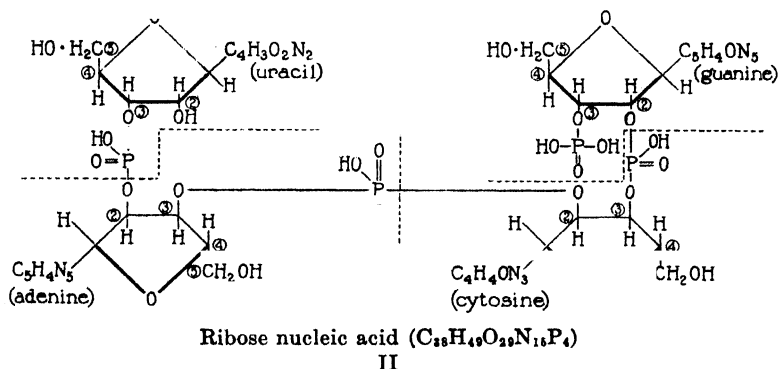
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furthermore, on hydrolysis with dilute mineral acids, yields pyrimidine nucleotides having one phosphoric acid radicle only and that attached in position (3), whereas desoxy-ribose nucleic acid under

<sup>4</sup>It is more likely that the phosphoryl residue connecting the thymine- and adenine-desoxy-ribosides is situated at positions (5) of their sugar chains and that the union from the adenine- to the cytosine-desoxy-ribosides is through positions (3).

similar conditions yields pyrimidine nucleotides with two phosphoric acid radicles, which must be in positions (3) and (5).

Inasmuch as the phosphoric acid radicle in 5-phospho-ribose is naturally quite resistant towards the hydrolytic action of dilute mineral acids, it is justifiable to assume that the hydroxyl in position (5) of *ribose nucleic acid* is not substituted and hence it is warranted to assign to this acid structure (II), in which the phosphoryl residues are attached at positions (2) and (3). This structure explains the behavior of this acid towards alkalis. Since substituents, even ether linkages, in position (2) are characterized by greater instability than in other positions, it is readily understood that nucleotides linked to one another through position



(2) of the sugar should dissociate with greater velocity, thus yielding nucleotides with the phosphoric acid radicle in the more stable position on carbon atom (3) of the ribose chain.

#### EXPERIMENTAL

The thymidine used in the following experiments had a melting point of  $184^\circ$  and the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.63^\circ \times 100}{2 \times 1.029} = +30.6^\circ \text{ (in water)}$$

It was soluble in cold absolute methyl alcohol, pyridine, glacial acetic acid, and water; fairly soluble in cold and soluble in hot absolute ethyl alcohol; very sparingly soluble in cold but fairly

soluble in hot acetone or ethyl acetate; insoluble in cold but very sparingly soluble in hot chloroform. It crystallizes from ethyl acetate in rosettes of needles.

*Preparation of Monotrityl Thymidine*—A mixture of dry, finely powdered thymidine (0.5 gm.) with pure, dry triphenylmethyl chloride (0.6 gm.) was dissolved in 15 cc. of dry, redistilled pyridine. The solution was allowed to stand, with the exclusion of atmospheric moisture, during 7 days at room temperature.

The solution, which was still clear and very pale yellow in color, was then poured into 100 cc. of ice water, with vigorous stirring. The pale yellow gum which was precipitated was twice washed with 100 cc. portions of ice water and allowed to stand under ice water during 3 days in the refrigerator. The gum had now changed to a hard, friable mass which was pulverized, filtered off, washed with water until free from pyridine, and air-dried.

The finely powdered product was now shaken with dry ether and the ether extract decanted. Pentane was added to the ether extract until no more precipitate formed. The precipitate was united with the ether-insoluble portion, dissolved in acetone, and the acetone solution evaporated to dryness under diminished pressure, giving a pale yellow glass (weight, 0.9 gm.). A further portion (0.1 gm.) was recovered from the ether-pentane filtrate in the following way. The solution was evaporated to dryness, giving a colorless crystalline mass which was dissolved in 5 cc. of dry ether. To this solution, 100 cc. of pentane were cautiously added, with shaking, and the flocculent precipitate filtered off.

The material was first obtained crystalline in the following manner. The glassy substance (1 gm.) was dissolved in 2.5 cc. of acetone and 100 cc. of dry ether were cautiously added to the solution, producing a small precipitate. The mixture was now evaporated under diminished pressure without a water bath. When the volume of the solution had been decreased to about 10 cc., the trityl thymidine commenced crystallizing spontaneously.

It was recrystallized by dissolving in a little acetone and adding dry ether. On nucleating and stirring it set to a solid mass of colorless crystals having a melting point of 125°. It was insoluble in cold or hot water; insoluble in cold but very sparingly soluble in hot pentane or heptane; sparingly soluble in cold but

slightly soluble in hot carbon tetrachloride; fairly soluble in cold but quite soluble in hot benzene; and quite soluble in cold dry ether, absolute ethyl alcohol, absolute methyl alcohol, acetone, chloroform, pyridine, ethyl acetate, and glacial acetic acid. It crystallized in rosettes of needles from hot benzene. It had the following composition.

3.910 mg. substance:	10.295 mg. CO <sub>2</sub> and 2.120 mg. H <sub>2</sub> O
5.590 " " :	0.281 cc. N <sub>2</sub> (762 mm. at 24°)
C <sub>20</sub> H <sub>24</sub> O <sub>4</sub> N <sub>2</sub> .	Calculated. C 71.87, H 5.8, N 5.79
	Found. " 71.80, " 6.0, " 5.78

It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.23^\circ \times 100}{2 \times 1.010} = +11.4^\circ \text{ (in acetone)}$$

*Preparation of Monotosyl Trityl Thymidine*—Dry, recrystallized trityl thymidine (0.4 gm.) was dissolved in 2.5 cc. of dry pyridine contained in a glass-stoppered 10 cc. Erlenmeyer flask, tosyl chloride (0.17 gm.) was added, and the mixture was shaken until the chloride had dissolved. After standing overnight at room temperature, with the exclusion of atmospheric moisture, the pale brown solution was cooled in ice and 2 drops of ice water were added.

The resulting solution was kept at room temperature during 30 minutes and then poured into 100 cc. of ice water with stirring. The white powdery precipitate was filtered off, repeatedly washed with ice water until free from pyridine, and dried overnight in the vacuum desiccator over phosphorus pentoxide and soda-lime.

It was soluble in cold absolute ethyl alcohol, absolute methyl alcohol, acetone, chloroform, carbon tetrachloride, benzene, pyridine, ethyl acetate, and glacial acetic acid; fairly soluble in cold or hot dry ether; insoluble in cold and very sparingly soluble in hot pentane or heptane; and insoluble in cold or hot water.

It had the following composition.

4.700 mg. substance:	11.670 mg. CO <sub>2</sub> and 2.185 mg. H <sub>2</sub> O
7.100 " " :	0.273 cc. N <sub>2</sub> (776 mm. at 25°)
13.200 " " :	4.860 mg. BaSO <sub>4</sub>
C <sub>26</sub> H <sub>34</sub> O <sub>7</sub> N <sub>2</sub> S.	Calculated. C 67.67, H 5.4, N 4.39, S 5.02
	Found. " 67.70, " 5.2, " 4.50, " 5.05, Cl 0.00

It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.60^\circ \times 100}{2 \times 1.009} = +29.7^\circ \text{ (in acetone)}$$

*Action of Sodium Iodide on Tosyl Trityl Thymidine*—A mixture of 100 mg. of dry tosyl trityl thymidine with 100 mg. of dry sodium iodide was dissolved in 2 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. The solution became very light brown in color and a small amount of flaky crystalline material was deposited. The crystals were filtered off, dried, and weighed. Weight, 10 mg. (about 30 per cent of what should have been formed, had the reaction proceeded to completion).

The acetone solution and washings were united and evaporated to dryness and the product isolated in the usual way,<sup>3</sup> giving a white amorphous powder having the following analysis.

3.926 mg. substance:	9.400 mg. CO <sub>2</sub> and 1.900 mg. H <sub>2</sub> O
C <sub>20</sub> H <sub>14</sub> O <sub>7</sub> N <sub>2</sub> S. Calculated.	C 67.67, H 5.4
C <sub>20</sub> H <sub>17</sub> O <sub>4</sub> N <sub>2</sub> I. " "	" 58.57, " 4.6
Found.	" 65.29, " 5.4, ash 0.00

In a second experiment in which 180 mg. of tosyl trityl thymidine were treated with sodium iodide in acetone at room temperature (18 hours), then at 100° (2 hours), and finally at room temperature (18 hours), the yield of sodium *p*-toluenesulfonate was 27 mg. (about 50 per cent of the theoretical). The product from the acetone solution had the following analysis.

4.667 mg. substance:	11.125 mg. CO <sub>2</sub> and 2.330 mg. H <sub>2</sub> O
4.997 " " :	1.095 " AgI
C <sub>20</sub> H <sub>17</sub> O <sub>4</sub> N <sub>2</sub> I. Calculated.	C 58.57, H 4.6, I 21.36
Found.	" 64.99, " 5.5, " 11.84

*Action of Tosyl Chloride on Thymidine*—Dry thymidine (0.5 gm.) was dissolved in dry pyridine (3 cc.) and tosyl chloride (0.9 gm., approximately 2.2 moles) was added. After standing overnight at room temperature with the exclusion of atmospheric moisture, 0.1 cc. of water was added to the brown solution. The resulting solution was kept at room temperature during 30 minutes and the product isolated by pouring into 100 cc. of filtered ice water with stirring. The pale pink powdery precipitate was filtered off and

washed repeatedly with ice water until free from pyridine. It was then dissolved in acetone and evaporated to a hard glass (1.0 gm.) which could not be obtained crystalline as it consisted of a mixture of ditosyl thymidine with some monotosyl chlorothymidine (about 25 per cent).

*Action of Sodium Iodide on Tosylated Thymidine*—A mixture of 0.6 gm. of crude ditosyl thymidine (containing monotosyl chlorothymidine) with 0.6 gm. of dry sodium iodide was dissolved in 5 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. The solution became brown in color and a large amount of crystalline material was deposited. The crystals were filtered off, dried, and weighed. Weight, 275.6 mg. The crystals were dissolved in water and the solution, diluted to 25 cc., had the following analysis.

10 cc. required 1.35 cc. 0.1 N  $\text{AgNO}_3$  (Volhard); found Cl 4.34

Hence the starting material contained about 25 per cent of monotosyl chlorouridine; and about 43 per cent of the secondary tosyloxy group was removed by sodium iodide in acetone.

The reaction product was isolated in the usual manner, giving a yellow, glassy material which had the following analysis.

7.190 mg. substance: 5.610 mg. AgI

$\text{C}_{17}\text{H}_{19}\text{O}_5\text{N}_2\text{IS}$ . Calculated. I 25.08

$\text{C}_{10}\text{H}_{12}\text{O}_5\text{N}_2\text{I}_2$ . " " 54.95

Found. " 42.17

### CORRECTIONS

On page 630, Vol. 109, No. 2, May, 1935, line 4 from foot of text, read *chlorothymidine* for *chlorouridine*.

## STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS

### XII. THE SOLUBILITY OF HUMAN HEMOGLOBIN IN CONCENTRATED SALT SOLUTIONS

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(Received for publication, March 14, 1935)

The precipitation of proteins by neutral salts has commonly been employed not only in their separation and purification but in their characterization. Separation is effected because some proteins are almost completely precipitated in a concentrated salt solution, in which others are relatively soluble. In the range in which a protein is precipitable we have previously (2, 6) shown that solubility is defined by an equation of the form

$$\log S = \beta - K' \frac{\Gamma}{2} \quad (1)$$

in which  $\Gamma$  is the sum of the concentrations of the ions multiplied by the square of their valence; that is,  $\Gamma/2$  is the ionic strength in moles per liter. The amphoteric properties of the protein are reflected by  $\beta$ , whereas  $K'$  is characteristic only of the salt and the protein, and appears to be independent of pH and temperature.

The physicochemical properties of the corresponding proteins of different species are not necessarily identical. It would therefore be extremely misleading to assume that because a given protein had been exhaustively studied the results were applicable to the analogous protein of another species. Some differences between the properties of the serum proteins of different species have been noted (1, 9). The solubility relations of the hemoglobin of horse (6) and man are, however, so different that



that of the human erythrocyte remains very soluble in a phosphate solution in which horse hemoglobin is essentially insoluble (Fig. 1). Human hemoglobin behaves much more like an albumin than does horse hemoglobin.

*Preparation*—The hemoglobin used in these experiments was prepared from normal adult blood. The citrated cells were separated from the plasma by centrifugation and the cells were then washed at least twice in hypertonic saline.

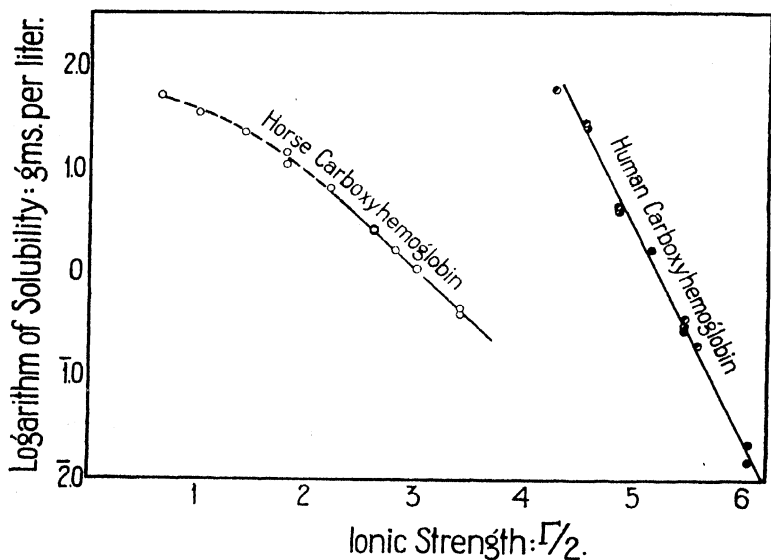


FIG. 1. Solubility of the carboxyhemoglobin of the horse and of man in phosphate buffers at 25°. Carboxyhemoglobin of horse  $\circ$ ; of man, Preparation 1  $\bullet$ , Preparation 2  $\bullet$ , Preparation 3  $\bullet$ .

In Preparations 1 and 2, made in 1931, water was added to lake the cells. At this point the solution was saturated with carbon monoxide, converting the hemoglobin to carboxyhemoglobin. The hemoglobin did not readily crystallize at the isoelectric point in dilute salt solution, although this is the procedure we generally employ in the purification of horse hemoglobin (5). Human hemoglobin readily crystallized, however, from a concentrated potassium phosphate buffer, the final concentration of phosphate being 2.8 M, the mole fraction of  $K_2HPO_4$  0.58, and the pH 6.8.

In Preparation 3, made in 1934, the cells were dialyzed in cellophane tubes after being washed in saline. After dialyzing against running water overnight, the solution was removed from the tubes and centrifuged to remove cell debris. The supernatant liquid was again returned to the dialyzing tubes which were then placed in the same phosphate buffer as was used in the other preparations, crystallization taking place as the salt dialyzed into the cellophane tubes, in the manner employed by Theorell (12) for muscle hemoglobin.

*Solubility*—Since solubility in concentrated salt solutions is a function not only of the concentration of the salt, but of pH and temperature, these studies, as those previously reported, have been carried out in phosphate buffers of known pH and ionic strength (3, 8). The crystalline carboxyhemoglobin was repeatedly triturated with fresh aliquots of the solvent for periods of from 12 to 48 hours at 25°. The saturated solutions were then filtered and analyzed for hemoglobin by nitrogen determination. Successive filtrates had essentially the same solubility for as long as a week. The results with the different preparations are in satisfactory agreement with each other, and in marked contrast with those of horse hemoglobin (6, 7). The behavior of these two carboxyhemoglobins is graphically represented in Fig. 1.

The maximum solubility of horse hemoglobin is 52 gm. per liter in a 0.33 M phosphate buffer at pH 6.6 and 25°. Human hemoglobin is as soluble as this in a 1.96 M phosphate buffer, but at this ionic strength the hemoglobin of the horse is only one-thousandth as soluble as that of man.

The solubility of horse carboxyhemoglobin obeys Equation 1 at ionic strengths greater than 2.0, human carboxyhemoglobin beyond 5.0. Indeed the deviation from a linear relation is not significant even in the most soluble systems studied. The constants for the straight lines in Fig. 1 are, in terms of Equation 1,

	pH	$\beta$	$K'_s$
Horse carboxyhemoglobin.....	6.6	3.0	1.0
Human " .....	6.8	10.4	2.0

The hemoglobins of different species, previously shown to have different crystal forms (11), different ratios of amino acids and of iron to sulfur (4, 13), and different solubilities in water (10), thus also have very different solubilities in concentrated salt solutions.

## SUMMARY

1. Human carboxyhemoglobin has been crystallized and characterized by its solubility in concentrated phosphate buffers.

2. Horse carboxyhemoglobin is precipitated at far lower ionic strengths than human carboxyhemoglobin, the latter behaving far more like an albumin.

3. The value not only of  $\beta$  but of  $K'$ , in the salting-out equation is far greater for human than for horse carboxyhemoglobin.

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## THE SURFACE INACTIVATION OF CATALASE

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(Received for publication, March 20, 1935)

Despite its importance and a vast amount of investigation concerning it, the exact mechanism of the rôle that surfaces play in enzyme chemistry is still comparatively unknown. Practically, in the separation and purification of enzymes, much use has been made of the adsorbing powers of such surface-active substances as charcoal, kaolin, ferric hydroxide, silicic acid, etc. (1). As a rule these adsorptive processes are more or less irreversible, and the adsorption curves were found by Kraut and Wenzel (2) to be diverse and anomalous. The use of such surface-active materials introduces a factor of chemical combination of the adsorbent with the enzyme. The adsorption is found to depend on the nature of the adsorbing material and the particular enzyme used (3). Consequently the study of adsorption as a physical process is not possible by the use of the above surface-active materials.

The adsorption or inactivation of enzymes by such relatively inert substances as glass or quartz has been the study of several investigators among whom are Beard and Cramer (4) who showed that glass beads have an inhibiting influence upon the activity of lipase, diastase, and invertase, and that a part, but not all of the activity, was regained when the beads were removed. They ascribed this inactivation to the alteration in concentrations produced by the change in surface energy at the glass surfaces. It is conceivable that if the enzyme is able to decrease the interfacial tension between the liquid-glass interface it would concentrate there and become inactivated by changing its state of dispersion (5). However, as glass is appreciably soluble in water and increases its alkalinity, Griffin and Nelson (6) demonstrated that glass beads cause a change in the reaction of an invertase extract

that is proportional to the number of glass beads used, and therefore the inactivation is due to a change in the hydrogen ion concentration, rather than to the surface effect of the beads; also that the beads produced no effect when the pH was kept constant by means of the buffers.

The author's attention was focused upon this problem during the course of some studies on the kinetics of the catalase-hydrogen peroxide reaction when it was observed that the presence of glass beads caused an inhibition of the reaction; this inhibition could be increased by adding more beads. It was also found that the catalase extract itself could be irreversibly inactivated by the addition of glass beads for a short time, the inactivation again being apparently proportional to the number of beads (7). Michaelis and Pechstein (8) had done this but reported no effect due to the addition of glass beads. Recently, Zeile (9), in attempting to determine the diffusion coefficient of catalase by passage through filters, found that the catalase was inactivated after going through the filter and considered it due to the fact that the enzyme when in solution dissociated, and that one of the components was of such a molecular size as to be unable to pass through the membrane, while the part that passed through was inactive. Assuming adsorption of catalase upon surfaces, it would appear that the pores of the filter offered sufficient surface area to allow adsorption and inactivation of the enzyme as it passed through the filter. In the hope of clearing up some of the points involved, and if possible, to obtain some quantitative data, it was decided to perform the experiments which are herein described.

#### *Procedure*

The enzyme extract was prepared by extracting a pound of ground fresh beef liver with a liter of chloroform water for 24 hours in the ice chest. The larger masses were removed by filtration through muslin; then by filtering twice through paper pulp a clear filtrate was obtained. This was added to an equal bulk of 95 per cent ethyl alcohol and the precipitated proteins were removed by centrifuging. The yellow, slightly opalescent filtrate represented the enzyme extract and when kept in the ice box appeared to retain its full strength almost indefinitely.

The glass beads used were of the ordinary laboratory grade, 3

mm. in diameter. These were prepared by washing in aqua regia for 24 hours, followed by thorough rinsing in tap water, and then in distilled water, after which they were dried by heat. Merck's superoxol was used for the hydrogen peroxide, small amounts of which were diluted to 0.25 M daily and titrated with standardized potassium permanganate before and after each day's experiments to detect any change in concentration of the diluted peroxide. Each 100 cc. of the hydrogen peroxide contained 15 cc. of Clark and Lubs' phosphate-sodium hydroxide buffer of pH 7.0 (10).

Titration of hydrogen peroxide with potassium permanganate in the presence of traces of organic matter from the added enzyme was found to be quite inaccurate, it being difficult to obtain good end-points. In our search for a volumetric method that would be unaffected by organic matter, titanous chloride ( $\text{TiCl}_3$ ) (11) was decided upon as previous experiences had proved it to be entirely unaffected by the presence of organic matter. The solution was kept under an atmosphere of hydrogen and delivered by means of an automatic burette. The titrations must be made as rapidly as possible. Standardization of the titanous chloride was by means of a hydrogen peroxide solution.

The catalase extract was inactivated by pipetting 15 cc. of the extract into large 50 cc. Pyrex test-tubes, each of which contained a quantity of the dried glass beads. Various weights, 1 to 100 gm., of the glass beads were used to furnish different amounts of surface area, assuming that if the beads were uniform in weight their combined areas could be represented by their total weight. The enzyme was allowed to remain in contact with the beads for 15 minutes at room temperature ( $24^\circ \pm 2^\circ$ ). Spontaneous temperature inactivation below  $50^\circ$  is insignificant (12). The pH of the enzyme (colorimetrically) before addition to the beads was 6.2 and this changed to 7.3 after the enzyme was in contact with the beads for 15 minutes. Evidently, enough of the glass dissolved to change the reaction of the enzyme extract which was used unbuffered so as not to introduce any salt effects. In contrast to Griffin and Nelson's (6) findings with invertase, the shift of the pH of the solution to 7.3 is in the direction of greater stability (12). Hence, we may disregard the change in pH as a factor in the inactivation, especially as there was very little variation with the amount of beads.

The amount of enzyme adsorbed was determined by pipetting 1.2 cc. of the catalase extract from the beads after the 15 minutes were over and adding this to 15 cc. of the 0.25 M buffered hydrogen peroxide and allowing the reaction to go to completion, which at 24° took about 1 hour. The amount of enzyme used was previously determined as the amount of enzyme that would decompose 15 cc. of 0.25 M peroxide to the extent of 80 per cent (13). Controls of the enzyme and of the hydrogen peroxide were run concurrently with the experiments. When the reaction was completed, 10 cc. of concentrated HCl were added and the hydrogen peroxide remaining was titrated with the titanous chloride.

As the amount of hydrogen peroxide decomposed is directly proportional to the concentration of active catalase added (14, 15), the increase in the quantity of hydrogen peroxide remaining undecomposed is directly proportional to the amount of enzyme adsorbed and represents the degree of inactivation of the catalase solution upon the beads. In Fig. 1, the relative areas of the glass beads are plotted against the percentage inactivation of the catalase. The average of all the results may be expressed by the curve as it is drawn. There appeared to be some suggestion of a logarithmic relationship and so Fig. 2, in which the percentage inactivation of the enzyme is plotted against the logarithm of the relative areas, was constructed. A direct relationship apparently holds for a portion of the graph, but there is a definite deviation in the region of the smaller areas, which may possibly be due to the relatively larger error in the surface area. There are many sources of error in a study of this type and until more precise measurements of surface area and enzyme activity can be obtained, together with a better control of experimental conditions, it is difficult to draw any more exact conclusions from the results.

#### *Adsorption of Catalase on Quartz*

It had been planned to repeat the experiments with fused quartz beads instead of glass ones, but as only a small quantity of the former were obtainable, the following experiment was performed.

5 gm. of transparent fused quartz beads were treated as were the glass beads and after being dried were placed in 10 cc. of catalase extract for 15 minutes at 24°. The pH of the enzyme solution was not changed by the quartz beads and remained at 6.2. The

enzyme was drained off and the beads were thoroughly washed with distilled water and alcohol and allowed to dry spontaneously. When dry, these beads were dropped into 10 cc. of 0.25 M hydrogen peroxide, whereupon an active decomposition of the hydrogen peroxide took place upon the surface of each quartz bead, from

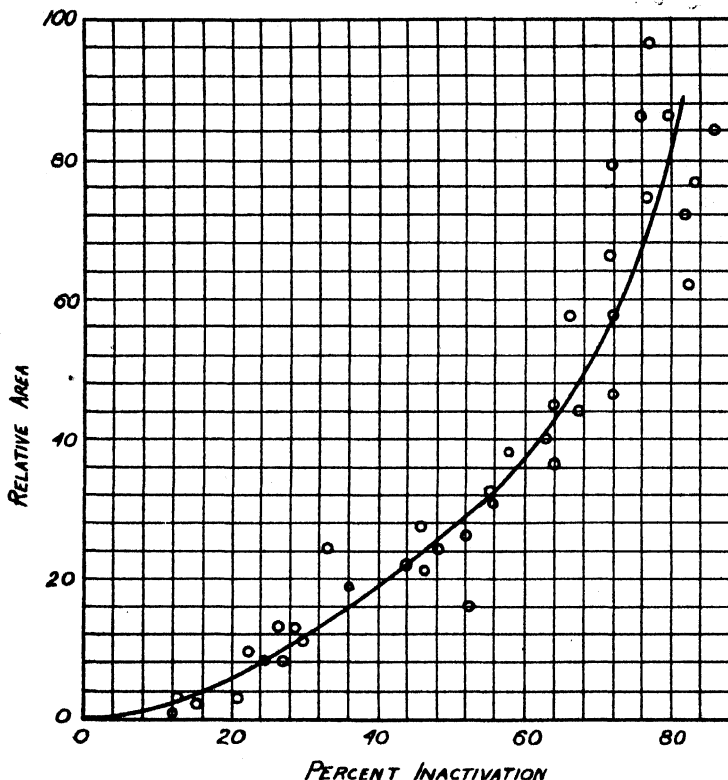


FIG. 1. Relation between the relative surface areas and the percentage inactivation of catalase.

which the bubbles of oxygen were seen to rise. A control quantity of quartz beads, similarly treated in all respects with the exception of the catalase, did not produce any decomposition whatsoever. As a phenomenon of adsorption this appears to be paradoxical, yet Abramson (16) has shown experimentally that practically all the



polar groups of a protein molecule may be available even after adsorption has occurred; *i.e.*, a protein may maintain full activity despite adsorption. He offers this as an explanation of the phenomenon observed by Beard and Cramer (4) and Griffin and Nel-

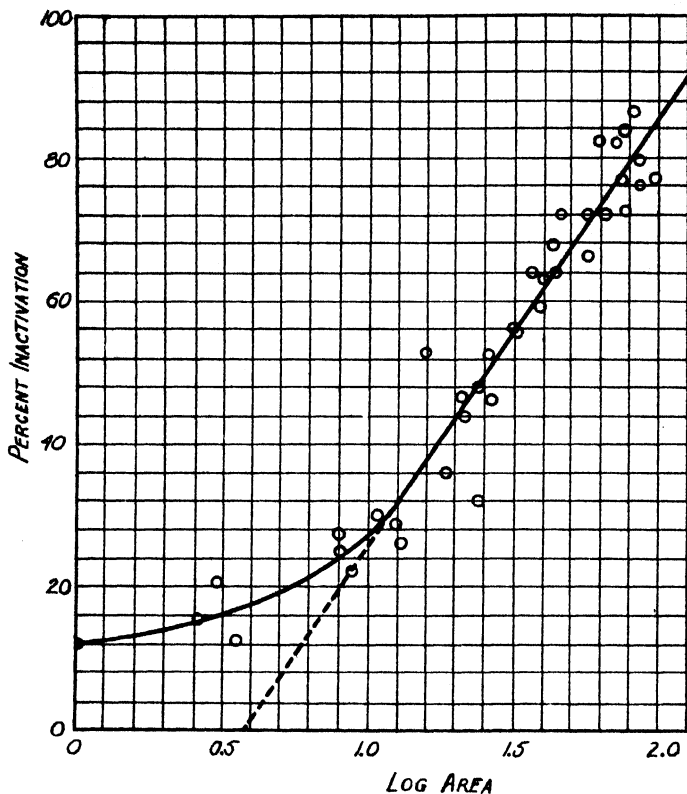


FIG. 2. Relation between the logarithm of the relative areas and the percentage inactivation of catalase.

son (6), that it is easily conceivable that enzymes that are protein-like in nature could be adsorbed on an inert or living surface and nevertheless suffer no diminution in their enzymatically active groups. It then also holds for the adsorption of catalase upon quartz, in which case the catalase molecule when in solution

apparently acts as a whole and does not dissociate, or if it does, then its component parts or ions must have the same adsorption affinity for quartz, and when concentration at the solution-quartz interface occurs, then association takes place.

#### SUMMARY

1. A catalase solution is inactivated in the presence of glass beads. The catalase probably is adsorbed upon the glass surfaces. Although the glass is soluble and changes the hydrogen ion concentration of the enzyme solution, the inactivation of the solution is thought due to the concentration of the catalase at the solution-glass interface with a resultant adsorption upon the glass surface (9). The inactivation appears to be related to the logarithm of the surface area.

2. Catalase is adsorbed out of solution upon quartz surfaces without loss of the enzymatic properties of the adsorbed catalase. The quartz is not soluble enough to produce any changes in the hydrogen ion concentration of the enzyme solution.

The writer herewith desires to express his appreciation to Professor Matthew Steel of the Department of Biological Chemistry of the Long Island College of Medicine and to Dr. Jacinto Steinhardt of the Laboratory of Biophysics of Columbia University. Their interest in the problem and their suggestions proved most valuable.

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# THE EFFECT OF CARBON DIOXIDE UPON THE pH AND CERTAIN NITROGEN FRACTIONS OF THE SUGAR-BEET PLANT

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It is the general belief that the hydrogen ion activity of the cell sap is increased by the accumulation of carbon dioxide. For example, Willaman and Beaumont (5) observed that when carbon dioxide was allowed to accumulate in an atmosphere in which twigs, tubers, or grain was stored, the rate of carbon dioxide production by these tissues decreased in a logarithmic ratio. In the case of the twigs, the amount of carbon dioxide produced was proportional to the logarithm of time. The rate of production of carbon dioxide immediately assumed a far higher value when the accumulated carbon dioxide was removed. In seeking an explanation, these investigators ((5) p. 52) state: "Another possible explanation was suggested by Dr. R. A. Gortner. It is that the accumulation of  $\text{CO}_2$  in the tissues increases the hydrogen-ion concentration in the latter; that this brings the proteins of the protoplasm nearer to their isoelectric point, and hence increases its permeability, which is responsible (perhaps through increased enzyme activity) for an actual increased rate of  $\text{CO}_2$  production." A further quotation from Willaman and Beaumont ((5) p. 53), "That the acidity of the tissue fluids is increased by the accumulation of  $\text{CO}_2$  is well known, and does not need a specific illustration," shows how generally this belief is held.

This idea is apparently supported by the fact that carbon dioxide increases the hydrogen ion activity of the expressed plant juice. The fact that the hydrogen ion activity of the cell sap within the living plant is not increased when the plants are exposed to high concentrations of carbon dioxide will be shown in this paper and an explanation of the reactions involved will be suggested.

Magness and Diehl (1) observed that in some cases the acidity of fruit was decreased on treatment with carbon dioxide. They also observed that the titratable acidity of the winesap apple, expressed as cc. of 0.1 N acid per 10 gm. net weight of tissue, decreased from 6.55 to 6.14 with a treatment of 100 per cent carbon dioxide.

Thornton (4) reported that the treatment of various types of plant tissues with carbon dioxide resulted in a decrease in the hydrogen ion concentration of the juice extracted from those tissues.

The studies on the effect of carbon dioxide reported in this paper were conducted in 1931 and 1932 in connection with investigations on the chemical nature of resistance in sugar-beets to the curly top disease. In these investigations pH measurements of the juice of various strains of sugar-beets and other plants affected by the disease were made. The first pH determinations were made with the quinhydrone electrode. Juice from the leaves of the various strains of sugar-beets (mass selections), and from individual plants of the same strains, varied in pH from 6.07 to 6.88. With the same electrode, leaf juice from *Chenopodium murale* (extremely resistant to curly top) consistently gave pH values varying from 7.3 to 8.0<sup>1</sup> immediately after extraction. In view of this apparent difference in pH and the greater disease resistance of the *Chenopodium murale*, it seemed probable that if the pH value of this plant could be temporarily reduced it might be rendered susceptible to the disease.

In view of the acid nature of carbon dioxide gas, an attempt was made to reduce the pH of *Chenopodium murale* by subjecting the entire plant to an atmosphere rich in this gas. When the plant was removed from the gas chamber and the juice expressed immediately, it was observed that the pH had not decreased as was

<sup>1</sup> Further pH determinations made at a later date revealed that these values, although constant, were high and misleading. If the juice extracted from *Chenopodium murale* were allowed to stand in the refrigerator for 3 days before pH determinations were made, true values were obtained. It is quite probable that certain soluble proteins are responsible for the abnormal results. It was observed that during storage a precipitate formed. True pH values were obtained (when precipitation was complete) even in the presence of the precipitate. Storage of the beet juice in the refrigerator had no effect on the pH value.

expected, but had greatly increased. This experiment was repeated several times with the same result. Further investigations revealed that sugar-beet plants responded in the same manner, the pH being increased as much as 2 units in some cases.

This unexpected decrease in the hydrogen ion concentration of the juice of these plants, when exposed to a high concentration of carbon dioxide, encouraged more detailed investigations into the nature of the chemical reactions involved because of a possible bearing on resistance to curly top.

### *Methods*

The plants receiving the carbon dioxide treatment were placed under bell jars and definite amounts of carbon dioxide were applied for different lengths of time at room temperature. The experiments were carried out both in the dark and in the light. Juice was extracted from the controls and from the carbon dioxide-treated plants with the aid of a hydraulic press immediately upon their removal from the bell jars.

The pH measurements were made with the glass electrode, the hydrogen electrode, and the quinhydrone electrode. The glass electrode used was similar to that described by Robertson (3).

### *Results*

In the first experiments, the entire plants were subjected to an atmosphere containing 50 per cent carbon dioxide for 24 hours. The plants were then removed and the juice expressed immediately. Table I shows the effect of a high concentration of carbon dioxide on the pH of the plant. These data are typical of more than 100 experiments which were conducted over a period of 2 years. In every case where the concentration of carbon dioxide was 10 per cent or greater, the pH of the juice, extracted from the plants immediately after treatment, was found to be higher than that of the juice from the untreated plants. In some of these experiments, the hydrogen ion activity of the juice of beet leaves was decreased as much as 100-fold by treating the plant with a high concentration of carbon dioxide. The results were of the same order whether the carbon dioxide exposure was made in the light or in the dark. When the entire plant was treated, juice extracted from the leaves, petioles, and roots showed an increase

in pH, as shown in Table I which gives the results of representative tests.

The pH of juice extracted from carbon dioxide-treated plants does not change on standing, yet it responds to carbon dioxide treatment in the normal way. It was found, for example, that when the expressed juice from carbon dioxide-treated beet leaves having pH of 7.45 was saturated with carbon dioxide, the pH decreased to 5.93. When the dissolved carbon dioxide was removed, the pH returned to its original value of 7.45. Juice expressed from normal beet leaves responded in a similar manner, going from pH 6.55 to 5.75 when saturated with carbon dioxide, and returning to its original pH of 6.55 when the gas was removed.

TABLE I

*pH of Juice Expressed from CO<sub>2</sub>-Treated and Normal Plants, As Determined by Quinhydrone Electrode*

Plant		pH of expressed juice	
		Normal	CO <sub>2</sub> -treated
<i>Chenopodium murale</i> Sugar-beet	Leaves	7.35*	8.11
	Blades	6.26	7.36
	Petioles	5.80	7.05
	Roots	6.11	6.57

\* See foot-note 1.

Neither macerated nor plasmolyzed tissues responded to carbon dioxide treatment as did the living plant; *i.e.*, by an increase in pH.

The fact that high concentrations of carbon dioxide will effect an appreciable change in the pH of other plants was demonstrated with several species of plants susceptible to curly top. In these tests the pH determinations were made with the glass, the hydrogen, and the quinhydrone electrodes, as shown in Table II.

All plants that have been tested thus far have responded in the same manner to carbon dioxide treatment. The degree to which the pH increases on carbon dioxide treatment varies widely and appears to be independent of the pH of the normal tissue. The chemical reaction responsible for this increase in pH is very effective in view of the buffering of the normal cell sap. To illustrate, the pH of *Oxalis martiana* was found to be 2.17 nor-

mally. This value was increased to 2.74 by treating the plant with carbon dioxide, despite the high buffer index (0.070) of the juice.

The pH values obtained with the glass electrode are probably more reliable than those with the quinhydrone or hydrogen electrodes, because the glass electrode is unaffected by oxidizing agents, catalysts, or poisons. From Table II it is evident that the quinhydrone electrode is suitable for pH measurements on the

TABLE II  
*pH of Expressed Juice of CO<sub>2</sub>-Treated and Normal Plants*

Plant	Electrode					
	Glass		Hydrogen		Quinhydrone	
	Normal	CO <sub>2</sub> -treated	Normal	CO <sub>2</sub> -treated	Normal	CO <sub>2</sub> -treated
<i>Asparagus officinalis</i> .....	6.09	6.51				
<i>Atriplex bracteosa</i> .....	6.17	6.43	6.14	6.72	6.47	6.80
<i>Beta vulgaris</i> .....	5.95	6.87	6.07	7.20	6.08	6.91
<i>Chenopodium murale</i> .....	6.39*	6.65	6.45*	6.89	6.36*	6.71
<i>Erodium cicutarium</i> .....	5.58	5.75	5.35	5.91	5.54	6.03
<i>Lycopersicon esculentum</i> .....					5.12	5.34
<i>Matthiola incana</i> .....	5.20	5.46	5.12	5.36	5.23	5.46
<i>Nicotiana tabacum</i> .....	5.25	5.52	5.22	5.45	5.36	5.66
<i>Oxalis martiana</i> .....	2.24	2.63	1.98	2.46	2.17	2.74
<i>Solanum nigrum</i> .....	5.60	6.10	5.59	6.13	5.36	6.05
<i>Stellaria media</i> .....	6.10	6.77	6.09	7.32	6.09	6.84

\* It is of interest to point out the close agreement in pH of *Chenopodium murale* as determined by the different electrodes. In this instance the juice was extracted and allowed to remain in the refrigerator for 3 days before the pH determinations were made with the quinhydrone electrode.

juice of the beet leaf and of other plants. In view of this fact, the subsequent measurements of the changes in pH on the juice of the beet leaf were made with the quinhydrone electrode.

Such striking changes in the hydrogen ion concentration of the plant juice, due to carbon dioxide treatment, must be the result of certain chemical reactions catalyzed by the plant. It appears logical that, if carbon dioxide or the increase in hydrogen ion concentration in the cell sap due to the dissolved carbon dioxide is the catalyst for these reactions, the rate of response of the plant



and the extent to which the reaction takes place will depend on the concentration of carbon dioxide dissolved in the cell sap and the duration of treatment.

In view of the fact that the diffusion of carbon dioxide into the leaves takes place through the intercellular spaces, the rate of diffusion of the gas and the amount dissolving in the cell sap depend on the partial pressure of carbon dioxide applied. A determination of the speed with which the hydrogen ion activity is altered in the plant (presumably counteracting the acidic effect of the carbonic acid formed in the cell sap) is of considerable importance in that it may throw light on the rate at which certain reactions proceed in plants.

In order to determine the rate at which beet plants will respond to carbon dioxide treatment, a large number of beet leaves were collected and divided into six lots, one of which was the control. The leaves were placed under bell jars and subjected to an atmosphere containing 40 per cent carbon dioxide. At the end of each of the following periods—30, 60, 90, 120, and 180 minutes—one lot of leaves was removed, the midribs and petioles discarded, the juice extracted immediately, and the pH determined.

Then, to determine the rate at which the beet plant will return to normal after carbon dioxide treatment, a large sample of beet leaves was placed under a large bell jar and exposed to an atmosphere containing 40 per cent carbon dioxide for 1 hour, after which it was removed. A small number of leaves were extracted immediately, while the remainder was allowed to stand in the laboratory. At 15, 30, 60, 90, and 180 minute intervals after their removal from the bell jar, the juice was extracted from a small number of leaves and the pH determined.

Curve A of Fig. 1 shows the rate at which the beet plant responded to carbon dioxide treatment, while Curve B shows the rate at which the pH of the plant returned to normal when the carbon dioxide treatment was discontinued. It is evident that the beet leaf catalyzes the reaction responsible for the pH change at a very rapid rate in either direction.

In view of the fact that only the partial pressure of carbon dioxide inside the cells would be effective in causing the plant to react in such a striking manner, a study of the effect of different concentrations of carbon dioxide on the reaction of the plant was made to determine how far the reactions would proceed.

A large number of beet leaves were collected and divided into seven lots, one of which served as the control. Each of the remaining lots of leaves was subjected to a definite concentration of carbon dioxide for 30 minutes. The samples of leaves were then removed from the bell jars, the juice extracted immediately, and the pH determined. The experiment was repeated, except that in this case each lot of leaves was allowed to remain in its

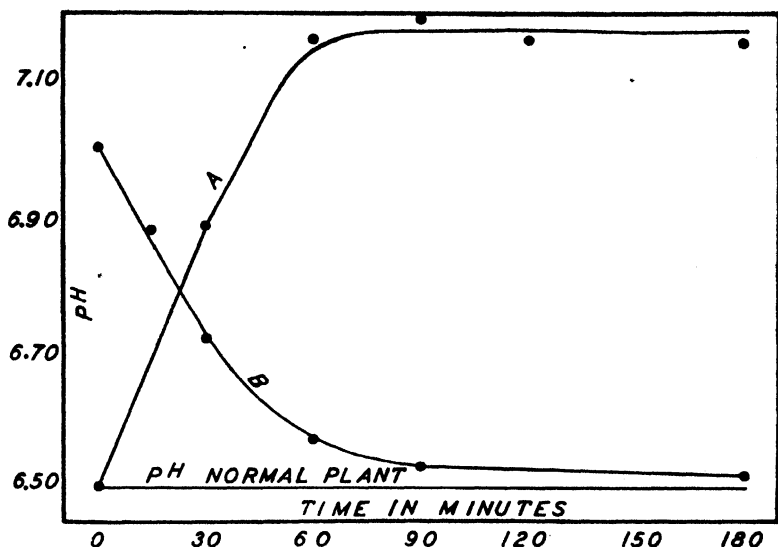


FIG. 1. The rate at which sugar-beet plants respond to and recover from carbon dioxide treatment. Curve A, the rate of response to carbon dioxide treatment; Curve B, the rate at which carbon dioxide-treated plants returned to normal.

respective concentration of carbon dioxide for a period of 1 hour. The results of these experiments are shown in Fig. 2.

It is apparent that the partial pressure of carbon dioxide is not the limiting factor in determining the extent to which the pH will change, for 20 per cent is almost as effective as 40 or 60 per cent in producing a change. At 80 per cent carbon dioxide (Fig. 2) the reaction is greatly intensified in both the 30 and 60 minute periods of treatment. This is very striking, especially when compared to the change in pH which occurs in 100 per cent carbon

dioxide. It is important to note that at 100 per cent carbon dioxide an increase in pH of the extracted juice was obtained only when the plants were exposed to the gas for a short period (approximately 1 hour). With an exposure of 4 or 5 hours the pH of the extracted juice was found to be considerably decreased below that of untreated controls.

The evidence tends to show that the changes which take place in the beet leaf during treatment with high concentrations of carbon dioxide are not due to translocation. Leaves or petioles

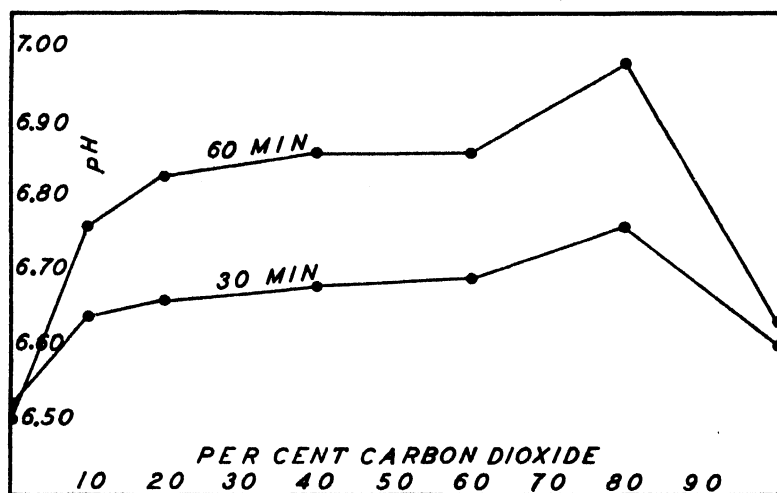


FIG. 2. The pH change in relation to the concentration of carbon dioxide applied to the beet plant.

which are removed from the remainder of the plant respond as readily and to the same degree to carbon dioxide treatment as leaves and petioles attached to the plant. The reactions, then, are local in character. However, this reaction is catalyzed by all parts of the plant. It is also evident that the reaction is not dependent on radiant energy, because the change takes place as readily in the dark as in the light.

In casting about for an explanation of this phenomenon and the possible reactions involved in the process, certain nutrition experiments in which beet plants received different amounts of nitrogen

furnished one clue to the type of compounds involved. It was observed that leaves taken from a series in which the plants received an abundance of nitrogen responded to a greater degree (as measured by the increase in pH on carbon dioxide treatment) than leaves removed from plants which were nitrogen-starved.

In view of these facts, it was suspected that perhaps the nitrogenous compounds in the plant were involved in the reactions which cause a decrease in the hydrogen ion activity of the plant. Experiments were carried out to determine what rôle the nitrogenous compounds play in these reactions.

A large number of beet leaves were collected, part of which was treated with carbon dioxide for a definite period, the remainder serving as a control. After the carbon dioxide treatment, the petioles and midribs were removed from the sample of leaves, and the juice was extracted from the blades. The juice was also extracted from the blades of the control leaves. The remainder of the carbon dioxide-treated leaves was left in the open for 1 hour, after which the juice was extracted from the blades. The pH was determined on the three lots of juice. Each lot was also analyzed for total soluble, ammonia, and amide nitrogen as outlined by Nightingale, Robbins, and Schermerhorn (2). The changes in pH, ammonia, and amide fractions resulting from the carbon dioxide treatment are shown in Table III.

No significant change in the water-soluble nitrogen (that fraction which is not coagulated by acid or heat) was found to occur in the plants during treatment. During the exposure to high concentrations of carbon dioxide, ammonia was split off from certain compounds in sufficient quantities to account in full for the increase in pH. It is evident from the data that ammonia is formed partly at the expense of the acid amides in all experiments. In Experiment 1 of Table III, the change in pH due to the treatment was from 6.00 to 7.20. The ammonia nitrogen increased from 3.52 to 11.00 per cent, or a net increase of 7.48 per cent. At the same time the amide nitrogen decreased from 16.43 to 8.50 per cent, a net decrease of 7.93 per cent. In other words, the decrease in amide nitrogen accounts in full for the ammonia formed in Experiment 1. Calculations show that the amount of ammonia formed will account in full for the decrease in the hydrogen ion activity observed. The data show that acid amides are hydro-

lyzed with the liberation of ammonia. The following equation suggests the reaction probably involved:  $2R \cdot CONH_2 + 3H_2O$  yields  $R \cdot COONH_4 + R \cdot COOH + NH_4OH$ . From the other two experiments, however, it is evident that ammonia is being split off from compounds other than acid amides. In Experiment 3, where the carbon dioxide concentration was 80 per cent, only 10 per cent of the ammonia came from the acid amides. This shows that the type of compounds furnishing the ammonia depends on

TABLE III

*Changes in pH, Ammonia, and Amide Nitrogen of Sugar-Beet Leaves Due to CO<sub>2</sub> Treatment*

Experiment No.	Blades from plants	Treatment		pH	N in H <sub>2</sub> O-soluble fraction (dry basis)		Gain in NH <sub>3</sub> -N	Loss in amide N	NH <sub>3</sub> -N coming from amides
		CO <sub>2</sub>	Time		NH <sub>3</sub>	Amide			
		per cent	min.		per cent	per cent	per cent	per cent	per cent
1	Untreated			6.00	3.52	16.43			
	Treated	20	90	7.20	11.00	8.50	7.48	7.93	100
2	Untreated			6.34	2.02	5.82			
	Treated	40	60	7.27	7.00	3.63	4.98	2.20	44
	“ recovered*	40	60	6.62	3.75	4.48			
3	Untreated			6.61	2.59	1.56			
	Treated	80	90	7.06	8.22	0.98	5.63	0.58	10
	“ recovered*	80	90	6.43	3.69	1.42			

\* These plants were allowed to remain in the laboratory only 1 hour before being extracted. According to results shown in Fig. 1, it requires a longer period for the plants to return to normal. It is quite probable that the ammonia and amide nitrogen would have returned to normal if a longer recovery period had been allowed.

the concentration of carbon dioxide to which the plants are subjected. When the plants were allowed to recover from the carbon dioxide treatment, the ammonium salts were reconverted to the acid amides and other compounds which were affected by the treatment with a production of hydrogen ions. Previous experiments indicated that if the carbon dioxide had been allowed to stand longer before the juice was expressed, the pH, and amide and ammonia nitrogen would have returned to normal.

## DISCUSSION

Certain plants, particularly sugar-beet, when subjected to a high concentration of carbon dioxide, catalyze certain reactions at a very rapid rate, which counteract an increase in the hydrogen ion activity of the cell sap. One of the catalytic reactions which apparently takes place in the beet plant, and which neutralizes the hydrogen ions formed, is as follows:  $R \cdot CONH_2 + 2H_2O$  yields  $R \cdot COOH + NH_4^+ + OH^-$ . The concentration of hydroxyl ions produced will depend on how far the reaction proceeds and the strength of the organic acids liberated. A survey of the dissociation constants of the different organic acids revealed that the amides of the amino acids must furnish a large portion of the ammonia to account for the increase in pH by the above reaction. It is evident from Table III, however, that ammonia is split off from other soluble compounds which may not be acidic in nature, such as free amino groups of soluble proteins.

This would account for the maximum increase in pH observed (Fig. 2) when the plant is subjected to 80 per cent carbon dioxide. When the plant is subjected to 100 per cent carbon dioxide, two of the reactions occurring in the leaf tend to counteract each other. In the first reaction, the plant produces hydroxyl ions which neutralize the carbonic acid in the cell sap. Where the oxygen tension is extremely low, comparatively strong organic acids form an important part of the end-products of respiration. These acids formed in respiration would neutralize the hydroxyl ions formed in the first reaction. Consequently, the pH of plants treated with 100 per cent carbon dioxide would depend on the length of time the plants were exposed to the gas. If the exposure is relatively short (the plants removed before the free oxygen is completely exhausted), the pH of the extracted juice would be greater than that of untreated plants. If, however, the plants are exposed to the pure gas for a long period, the organic acids formed in respiration would mask the former reaction and cause the hydrogen ion concentration of the cell sap to become higher than normal.

When carbon dioxide-treated beet plants or leaves are allowed to recover, the amide nitrogen increases to its original value apparently at the expense of the ammonia nitrogen with the formation of hydrogen ions. The reaction may be shown as follows:  $R \cdot COOH + NH_4^+$  yields  $R \cdot CONH_2 + H_2O + H^+$ .

It is well known that the juice from the leaves of such plants as the sugar-beet, tomato, *Chenopodium murale*, and others, is very poorly buffered at their normal pH. Despite the extremely low buffer index of the juice of the beet leaf (0.0048 between pH 6 and 7), the plant is able to maintain its normal pH when subjected to extremely adverse conditions. It seems logical to suppose that the plant complex is so arranged that the normal pH could be maintained by means other than the buffer capacity of the cell sap. The above reactions apparently accomplish this purpose in the beet plant, for the reaction proceeds in the beet plant in either direction at a very rapid rate, producing hydrogen or hydroxyl ions according to the conditions imposed.

It is possible that further studies on the reactions catalyzed by the beet plant when under the influence of high concentrations of carbon dioxide might shed light on the reactions involved in the formation of amides, amino acids, and proteins in plants.

#### SUMMARY

Analyses of juice expressed from sugar-beet plants immediately after treatment with high concentrations of carbon dioxide show that striking chemical changes have taken place in the cell sap of the tissues.

Certain reactions which are catalyzed by the beet plants, exposed to high concentrations of carbon dioxide, prevent enormous increases in the hydrogen ion concentration of the cell sap. When the juice is expressed from the beet plant immediately after treatment with carbon dioxide, a determination of the increase in pH over that of normal juice indicates to what extent these reactions took place.

When the beet plants are exposed to a high concentration of carbon dioxide, ammonia is split off from acid amides presumably according to the reaction,  $2R \cdot CONH_2 + 3H_2O$  yields  $R \cdot COONH_4 + R \cdot COOH + NH_4^+ + OH^-$ .

The increase in ammonia nitrogen found in the juice accounts in full for the observed increase in pH. When high concentrations of carbon dioxide were applied to the plant, ammonia nitrogen was found to have been split off from soluble nitrogenous compounds other than the acid amides.

Beet plants respond rapidly to carbon dioxide treatment. A

significant increase in pH of the extracted juice was obtained after 5 minutes exposure to the gas, and a maximum pH was reached in approximately 1 hour. Recovery from carbon dioxide treatment (a return to initial pH) was found to be almost as rapid. The beet plants recovered in about 2 hours after removal of the plants from the gas chamber.

The juice expressed from carbon dioxide-treated beet plants is stable with respect to pH. The catalytic agents which accelerate the reaction or reverse the process are active only in the organized plant. This increase in pH as a response to carbon dioxide exposures appears to be a general type of response, for ten other species of plants were found to respond in a similar manner.

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# THE EFFECT OF ORGANIC DIETARY CONSTITUENTS UPON CHRONIC FLUORINE TOXICOSIS IN THE RAT\*

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There is a growing conviction among investigators that fluorides inhibit carbohydrate metabolism. Indeed there are ample experimental data to support this conviction. Lipman (6), Loebel (7), Seller and Janey (9), Embden and Lehnartz (4), and others have shown that fluorides inhibit lactic acid formation. Dickens and Simer (2) found that fluoride inhibits glycolysis in tissues. Since NaF showed little effect upon the formation of lactic acid from methylglyoxal, they took this observation as *prima facie* evidence that the point of attack of the fluoride was somewhere in the catabolism of the hexose to the triose stage. More recently the researches of Embden, Deuticke, and Kraft (3), and Myerhoff and McEachern (8) have shown conclusively that pyruvic acid arises from phosphoglyceric acid and that pyruvic acid is a normal glycolytic intermediate on the path to lactic acid. Thus fluorides decreased lactic acid formation by specifically inhibiting the conversion of phosphoglyceric acid to pyruvic acid. The major evidence indicates clearly that fluorides interfere with glycolysis.

Since fluorides inhibit carbohydrate metabolism—specifically, the formation of lactic acid—perhaps a shift in the balance of metabolites in the body might in some degree alleviate the toxicity of fluorine. Normally carbohydrates in the diet are considered to be the principle source of energy metabolites. If the mechanism involved in the utilization of these metabolites could be minimized by keeping the carbohydrate portion of the diet ab-

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normally low, and if an excess of metabolites from dietary fat be given, would it be possible to minimize fluorine toxicosis? Further, would the inclusion of lactates, lactic acid, or glycerol in part at least correct the inhibitory effect of NaF upon lactic acid formation? In order to seek an answer to these questions, a study was made to find the influence of certain organic dietary constituents upon the development of chronic fluorine toxicosis in the rat.

#### EXPERIMENTAL

An adequate ration planned to carry very little carbohydrate was used. Three 3-carbon chain compounds at 9 per cent levels were added to the ration in some lots to determine their influence upon the effect of fluorine. The ration (No. 34) used in this experiment was made up as indicated.

##### *Ration 34*

Commercial casein.....	40 parts
Lard.....	25 "
Butter oil.....	15 "
Yeast.....	4 "
Liver residue.....	2 "
Cod liver oil.....	1 "
Salt mixture.....	5 "

The performance of rats on this ration was checked against the performance of rats fed basal Ration A previously described (5). Ration A was a natural grain ration which had proved satisfactory for the study of chronic fluorine poisoning in rats for five generations.

The salt mixture used herein was compounded to conform to the principle mineral constituents of milk based upon Babcock and Russell's analyses (1). The salt mixture was composed of:

	<i>gm.</i>		<i>gm.</i>
NaCl.....	335	Fe(C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ) <sub>2</sub> ·6H <sub>2</sub> O.....	55
K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O.....	845	KI.....	1.6
Ca <sub>2</sub> H <sub>2</sub> (PO <sub>4</sub> ) <sub>2</sub> ·4H <sub>2</sub> O.....	190	MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	0.7
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	204	ZnCl <sub>2</sub> .....	0.5
CaCO <sub>3</sub> .....	600	CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	0.6
Total.....			2232.4

The Ca:P ratio of this salt mixture was approximately 1.9:1.0. When 40 per cent of the ration was made up of casein, the ratio

was shifted to 1.04:0.92. When this salt mixture was fed at 5 per cent of the ration, a daily feed intake of 10 gm. approximated the mineral intake in 50 cc. of whole milk mineralized with Fe, Cu, Mn, and Zn according to the practice of our laboratory.

Vigorous young growing rats weighing from 60 to 90 gm. were divided into eight lots with seven animals per lot. Each lot was

TABLE I  
*Feeding Plan for Various Lots of Rats*

Lot No.	Ration			
I	Basal Ration A (natural grain ration)			
II	"	"	"	+ 0.2% NaF
III	"	"	34	(high fat ration)
IV	"	"	34	+ 0.2% NaF
V	Ration of Lot IV with 9 parts Ca lactate added			
VI	"	"	IV	" 9 " lactic acid "
VII	"	"	IV	" 9 " glycerol added
VIII	"	"	IV	" 9 " equal parts K and Na lactate

TABLE II  
*Average Daily Fluorine Ingestion Expressed in Mg.*

Wk.	Lot II (0.09 per cent F)		Lot IV (0.09 per cent F)		Lot V		Lot VI		Lot VII		Lot VIII	
	F daily	F per kilo body weight	F daily	F per kilo body weight	F daily	F per kilo body weight	F daily	F per kilo body weight	F daily	F per kilo body weight	F daily	F per kilo body weight
1	6.0	77.8	2.7	34.8	3.8	44.7	4.9	56.0	3.3	38.8	3.6	42.2
2	6.0	77.8	2.8	34.2	3.4	36.6	4.4	44.0	2.9	32.5	3.6	39.8
3	6.0	77.8	5.7	62.0	7.3	66.6	6.7	57.6	6.2	59.6	6.5	64.5
4	6.2	83.9	5.7	59.5	4.6	37.3	6.6	54.5	4.9	47.8	5.5	51.6
5	6.3	84.0	7.3	56.6	5.6	38.9	5.9	46.3	4.7	40.5	3.9	33.8
6	6.0	80.0	5.5	36.8	7.2	46.8	7.2	56.9	4.9	39.1	3.4	28.2

made up of four males and three females, except in Lots VII and VIII where four females and three males made up the lots respectively. The allotment and feeding plan are given in Table I.

### Results

It is evident from the data presented in Table II that the quantity of fluorine ingested on Ration 34 was considerably less than

that ingested by the lot on basal Ration A with fluorine. The rations containing the lactic acid and the Na and K lactate mixture were slightly less palatable. Apparently 6 to 7 mg. of F daily was all the fluorine that the growing rat would tolerate. The energy content of Ration 34 allowed the animals to survive on a smaller daily feed intake than those on Ration A. Thus better growth and health were attained because the daily dose of fluorine was considerably reduced. Data from Lot II show that a daily

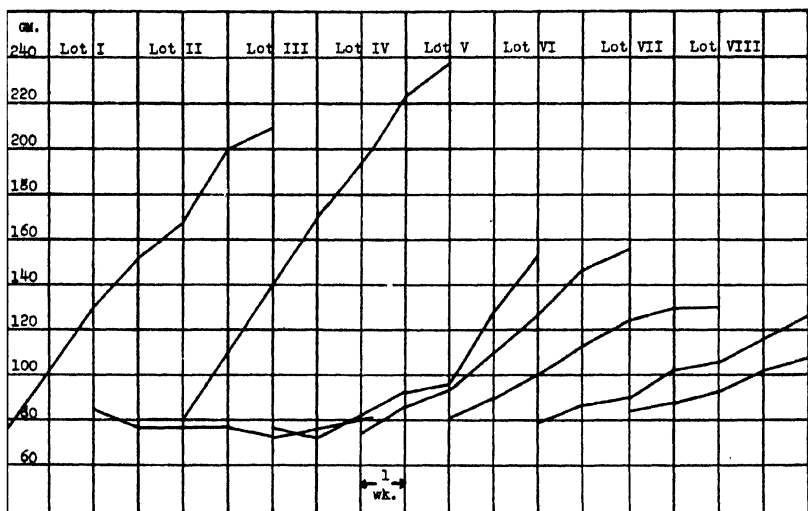


FIG. 1. Growth curves showing the effects of fluorine on rats fed a natural grain ration, or a high fat-low carbohydrate ration. For the feeding plan of the various lots of rats see Table I.

fluorine intake of 78 to 84 mg. of F in the form of NaF completely inhibited growth. Ration 34 permitted fluorine intakes much below this level during the 1st weeks of the experiment. The addition of lactates, glycerol, or lactic acid did not increase the tolerance to fluorine nor diminish the severity of its action. While Ration 34 was conducive to greater growth, as shown by Fig. 1, the superiority of this diet was believed to lie in its greater energy content per unit weight. Thus a high fat-low carbohydrate diet did not lessen the chronic toxic effects of fluorine

except as the higher energy content allowed a lower intake of fluorine per unit of body weight.

Observations upon the general health and gross symptoms of extreme chronic fluorosis in the growing rat show unmistakable signs of infancy. Infantile characteristics were manifest in the fur and genitalia. The fur failed to lose its fur-like character for the mature hairy coat, while the genitalia of both males and females did not develop. The state of extreme chronic fluorosis in the rat was that of arrested growth. Five of the rats from Lot II were continued after the close of the experiment and placed on Rations A and 34 without added fluorine. On these diets the body weight taken after a 48 hour fasting period doubled in 15 days. Activity was manifested in the genitalia by the opening of the vaginal orifice and by growth and descension of the testicle. The teeth, however, failed to return to normal in a recovery period of 60 days.

#### DISCUSSION

The evidence obtained in this study seems to indicate clearly that a shift in the balance of metabolites in the body does not alleviate the toxicity of NaF. Furthermore glycerol, lactic acid, or lactates at 9 per cent levels in the diet did not materially inhibit the development of fluorine toxicosis. These data point to the assumption that chronic fluorine poisoning involves more than the mechanism responsible for carbohydrate metabolism. Unless the catabolism of all excess protein and fat passes through the phosphoglyceric acid-pyruvic acid stage to the formation of lactic acid, it would not seem likely that Ration 34 would be without influence upon the development of fluorine toxicosis. It is, of course, conceded that part of the protein in its oxidation passes through the pyruvic acid stage. The oxidation of fatty acids has long been generally accepted as an oxidation taking place at the  $\beta$ -carbon atom with the ultimate formation of carbon dioxide and water from the intermediate acetic acid. If this theory of fatty acid oxidation is correct, then the oxidation of fats would avoid the specific fluorine-inhibited step involved in carbohydrate catabolism.

The relationship of chronic fluorine poisoning to growth seems to support the view that fluorine toxicosis is a generalized systemic

reaction. The fact that growth was resumed when fluorine was taken from the diet indicates a direct inhibition of the factors controlling the growth impulse. This action could be accomplished either through an influence upon certain endocrine glands, or directly by loss of appetite resulting in a lower feed intake and ultimate cachexia amounting to starvation. It seems unlikely that the effect of fluorine is upon the digestive tract directly since fluorine injections will cause lack of appetite and subsequently lower feed consumption. The phenomena of severe subminimal nutrition will produce many of the general symptoms noted; *i.e.*, infantile characteristics of fur and genitalia as well as inhibit growth. Several explanations seem plausible. Some of them are: that the fluorine influences directly the hormone control of growth through the enzymes involved in their action; that the cachexia reduces the available energy to a very low level of maintenance; and lastly one or more nutritional constituents may be reduced to sub-functional levels either directly through insufficient quantities being ingested caused by the reduction of feed intake, or through an interference with the function of the nutritional constituent at the site of utilization, the cell. It appears from this study and others made in this laboratory that the mode of action of fluorine is systemic in character, and it seems that this reaction is produced by its rather general inhibition of enzymatic systems. This view of the mode of action of chronic doses of fluorine in the animal organism stresses its influence upon the enzymes of the body rather than its specific effect upon Ca metabolism or the formation of lactic acid in the breakdown of carbohydrates.

The isolated specific effects of fluorides represent the mode of action of fluorine in part only. They, too, can be explained on the basis of the action of F upon the enzymatic systems involved. The possibility of fluorine being primarily associated closely with phosphorus in the animal economy makes it not unlikely that fluorine may interfere with, or disturb the actively metabolizing systems involving phosphoric acid esters.

#### SUMMARY

It has been shown that an intake of 78 to 84 mg. of F per kilo of body weight when ingested as NaF with a natural grain ration

will completely inhibit growth in the growing rat. Young growing rats would not tolerate more than 6 to 7 mg. of F per day.

A high fat-low carbohydrate diet did not ameliorate the toxicity of fluorine except as the high energy content allowed lower daily intakes of feed and consequently lowered the daily intake of F sufficiently to permit some degree of growth. The addition of lactates, glycerol, or lactic acid to Ration 34 (a high fat-low carbohydrate diet) did not increase the tolerance to F nor diminish the severity of its action.

These data are interpreted to mean that chronic fluorine poisoning involves more than a mechanism responsible for carbohydrate metabolism. The mode of action of F, viewed as a systemic reaction involving enzymatic inhibition, is discussed.

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# THE DETERMINATION OF THIOL AND DISULFIDE COMPOUNDS, WITH SPECIAL REFERENCE TO CYSTEINE AND CYSTINE

## I. CRITICAL STUDY OF THE COLOR REACTION BETWEEN PHOSPHO-18-TUNGSTIC ACID REAGENT AND THIOL COMPOUNDS

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The biological importance of organic sulfur compounds has led a number of workers to devise methods for the quantitative determination of thiol and disulfide compounds. The existing methods can be classified into the following seven groups: (1) nitroprusside (1, 2); (2) iodate or iodine (3-6); (3) phospho-18-tungstic acid (7-9); (4) naphthoquinone sulfonate (10-13); (5) precipitation by cuprous oxide (14, 15); (6) cobalt complex (1, 16, 17); (7) dimethyl-*p*-phenylenediamine (18).

Evaluation of these analytical methods depends chiefly upon (a) specificity, (b) precision, and (c) simplicity.

The first method in the form modified by Shinohara and Kilpatrick (2) is specific for water-soluble thiol compounds and is also simple, but lacks precision, and therefore is applicable only for rough estimation. The second and its modifications, regardless of their popularity, are not specific, nor precise. The third has been much used and modified. Its non-specificity and impermanency of color have frequently been criticized. However, it is with a modification of this method that the present paper deals. The fourth was claimed by the originator to be highly specific and its application in the biochemical field has been increased through a few modifications. Besides the large errors inherent in its complexity, which have recently been reported by Bushill *et al.* (13),

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the impermanency of the color and the great influence of the component reagents upon its intensity make the method less useful. Moreover, in spite of its claimed specificity, it utterly fails to differentiate cysteine from cystine, which is important for some biochemical studies. The fifth, besides being tedious, fails to differentiate thiol from disulfide compounds although it appears to determine their combined amount more or less accurately. The sixth is simple and specific for water-soluble thiol compounds, but the accuracy is not high, the average error occurring in color standards being roughly 3 per cent. Besides, the presence of histidine somewhat interferes with the determination. The seventh method has recently been worked out by Toyoda (18) at the author's suggestion. It is still in an unfinished state, and its applicability is doubtful.

Evidently none of these methods possesses all three essential qualities. This lack has caused neglect of standardization of thiol compounds, especially cysteine, even though it has been frequently used as the standard for colorimetric determination or for various other quantitative experiments. In many cases reported commercial cysteine was used seemingly with the assumption that it is of 100 per cent purity,<sup>1</sup> which is far from the truth, as will be shown in a later paper. In other cases, a cysteine solution obtained by reducing cystine with zinc or tin was used as a standard, assuming 100 per cent reduction. However, the reduction of cystine by zinc is such a slow process, even in a strong acid solution and at high temperatures, in spite of the rapid production of hydrogen gas, that complete reduction usually takes more than 24 hours. Tin, except in powder form, also reduces cystine very slowly. These facts indicate that serious errors have probably been committed.

The chaotic state of thiol and disulfide determination indicates the importance of establishing a method combining the three qualities. After trials with various methods, Lugg's study (9) of the phospho-18-tungstic acid method suggested many promising points toward such a purpose, in spite of its shortcomings in some aspects.

Therefore a series of critical studies was carried out, mainly

<sup>1</sup> Michaelis and Yamaguchi (17) actually determined the purity of cysteine used in their experiment by iodine and oxygen uptake methods.

from a pragmatcal standpoint. The conditions best suited for the determination of cysteine and cystine were established; a method was found for the easy standardization of cysteine and some other thiol compounds; and a method was found which can advantageously be used for urine and other biological materials. It was found by the latter method that fresh urine contains a thiol compound which is oxidized in air. These aspects will therefore be reported in a series of papers.

#### EXPERIMENTAL

##### *Reagents*

1. 0.01 M cysteine solution in 0.2 M HCl. A definite amount of cysteine hydrochloride (Pfanstiehl), of which the cysteine content had been determined by the method to be reported in a later paper, was used to make the solution. Cysteine hydrochloride is somewhat hygroscopic, increasing its weight exponentially on being exposed to the air. Therefore, the sample should be weighed within 5 minutes, allowing not more than 0.1 per cent increase. The solution should be discarded after standing over 2 days or its content should be redetermined.<sup>2</sup>

2. 0.01 M thioglycolic acid solution in 0.2 M HCl. An Eastman Kodak Company preparation was distilled under 18 mm. of Hg, and the middle portion that came out between 115–116° was collected. 0.2303 gm. was dissolved in 25 cc. of 2 M HCl and the total volume was made to 250 cc.<sup>3</sup>

3. Phospho-18-tungstic acid reagent. The reagent was made in

<sup>2</sup> This cysteine solution changes gradually on standing, mainly owing to oxidation, according to the polariscopic and the phospho-18-tungstic acid method, no H<sub>2</sub>S having been produced, even after 52 days. Although the rate of oxidation varies somewhat with the conditions, the cysteine concentration of the solution kept at 20–30° for a month can be expressed by the following equation, within the average error of about  $\pm 4$  per cent:  $C = C_0 (1 - 0.0023 N)$  where  $C_0$  is the initial concentration of cysteine (0.01 M), and  $C$  is the cysteine concentration after  $N$  days preservation.

<sup>3</sup> This purified thioglycolic acid was assumed to be practically 100 per cent pure, owing to the following facts: 1 mole of it combines with 0.5 mole of HgCl<sub>2</sub>; and, as will be shown later, the same molar concentrations of thioglycolic acid and cysteine produce the same color intensity. The stability of the thioglycolic acid solution was not tested, but in each experiment a solution not more than 5 hours old was used.

strict accordance with the directions given by Folin and Marenzi (8), except that the addition of lithium phosphate in the final stage was entirely omitted, being wholly unnecessary under the conditions employed by the author. The reagent thus prepared gives no color with phenol, tyrosine, tryptophane, other amino acids, and uric acid; and 1 cc. (roughly  $3.5 \times 10^{-4}$  moles as  $\text{WO}_3$ ) corresponds approximately to  $2 \times 10^{-5}$  moles of cysteine (2 cc. of 0.01 M cysteine solution).<sup>4</sup> 1 cc. of the reagent of the above strength will be called 1 unit of reagent.

4. The various other substances will be described as mentioned.

### *Technique*

Unless otherwise mentioned, the blue color due to the reduction of phospho-18-tungstic acid is developed in the following manner: 10 cc. of 2 M sodium acetate, 3 cc. of 2 M acetic acid solution, and an indefinite amount of water are first put into a 50 cc. glass-stoppered volumetric flask. Usually following the addition of a definite amount of the cysteine solution or a solution of other substances, 4 cc. of the reagent are added to the buffer solution and the total volume of the reaction mixture is made exactly 50 cc. When more than 3 cc. of the cysteine solution in 0.2 M HCl or a large amount of any other acid solution is added, the acid is neutralized by adding an equivalent amount of NaOH solution. Thus the pH of the solution is maintained in the close neighborhood of 5.0 and its composition is 0.4 M in sodium acetate, 0.12 M in acetic acid, 80 cc. per liter in the reagent, and varying concentrations in other compounds.

Immediately after it is made up to 50 cc., the flask is stoppered and shaken vigorously. The color intensity, which usually reaches the maximum within a few minutes, is measured after 5 to 10 minutes, unless a kinetic measurement is made.

The room temperature varied  $\pm 2.0^\circ$  at the maximum for a single experiment. For all the experiments the temperature range was  $27^\circ \pm 6^\circ$ .

<sup>4</sup> Although this equivalence is not important for the analytical method, it is convenient in determining various concentrations of cysteine and cystine. Therefore, the reagent prepared should comply with the above statements. The solution was found to be stable for at least 5 months.

*Standard Color Solution*—The color intensity of the mixture containing 2 cc. of 0.01 M cysteine<sup>5</sup> in 0.2 M HCl per 50 cc., besides all the other components mentioned under "Technique," was taken all through the experiments as the unit. The cysteine concentration of the standard color mixture, therefore, is  $4 \times 10^{-4}$  M (48.4 mg. per liter).

Six color standards were made from six different cysteine solutions and six colorimetric readings were taken of each of the color standards. The probable deviation of a single reading of the same color standard was found to be  $\pm 0.2$  per cent, while the deviation of a single reading from the average of all thirty-six readings was  $\pm 0.37$  per cent.<sup>6</sup> Such a small probable error has a great advantage over other methods. Moreover, the color of the mixture reaches its maximum in 2 to 3 minutes and remains constant for at least 6 hours in the room ( $t = 25^\circ \pm 3^\circ$ ). The change in intensity of the color standard solution was examined by comparing standard solutions of definite ages with standards freshly made for each determination.

A more detailed explanation of the color change of the standard will be seen in Table I.

*Influence of pH on Color Development*—Whether citrate or acetate is used as a buffer substance makes no material difference in the color intensity developed, as long as the pH of the mixture is the same; and the color intensity *versus* pH curve given by Lugg (9) was found to be correct, except for a slight disagreement in the part where pH is lower than 4.7. When phosphate buffer is used, phospho-18-tungstic acid is gradually precipitated, and the color developed by cysteine is less than might be expected. However, if the reagent is added after cysteine, practically the same intensity seems to be obtained.

The relation between color intensity and pH was carefully

<sup>5</sup> It was found by this colorimetric test that cysteine in the acetate buffer, in aeration, is stable for 2 hours, and oxidized 7 per cent at 4 hours, 50 per cent at 27 hours, 100 per cent at 70 hours.

<sup>6</sup> Where this color standard is used for color comparison with very dilute cysteine solutions, somewhat larger errors occur, as will be discussed later. Therefore, one containing  $4 \times 10^{-5}$  M cysteine may advantageously be substituted for it when a test solution contains less than  $1 \times 10^{-4}$  M cysteine. The color intensity of this solution is taken as 0.1 unit.

studied in the pH range of the acetate buffer,<sup>7</sup> reaction mixtures being used which contained the same amounts of components as described under the color standard, except for varying amounts of acetic acid. The time of the addition of the reagent was taken as the initial time. The pH was determined by color comparison, with solutions which contained all of the compounds but cysteine. When the amount of acetic acid added is small, the pH thus determined differs appreciably from the values calculated from the results of Larsson and Adell (19) ( $K_c = 4.52$  at 0.4 M) owing to the acidity of the reagent. Fig. 1, in which the results of such experiments are plotted, shows that the higher the pH of

TABLE I

*Change in Color Intensity of Color Standard Solution*

Composition: sodium acetate 0.4 M, acetic acid 0.12 M, cysteine  $4 \times 10^{-4}$  M, HCl  $8 \times 10^{-3}$  M. The reagent = 4 cc. per 50 cc.; pH 5.0; total volume 50 cc.

Time elapsed, hrs.....	0.1	2	4	6	24	30	50	79	102
Color standard kept in room ( $t\ 25^\circ \pm 3^\circ$ )		0.998 1.00	0.990 1.00	1.002 1.00		0.938 0.931	0.838 0.845	0.700 0.701	
Color standard kept in dark room ( $t\ 25^\circ \pm 3^\circ$ )	1.00			1.00		0.931		0.716	
Color standard kept in ice box ( $t\ 0-2^\circ$ )	1.00					0.995		1.005	

the medium, the faster is the color development, until the medium reaches pH 4.7, beyond which the maximum color intensity is reached within 2 minutes. In Fig. 2, the maximum intensities are plotted against the pH of the mixture. It shows that between pH 4.7 and 5.4, in which the amount of acetic acid added was varied from 5 to 1 cc., the color intensity remains the same. With

<sup>7</sup> The author used acetate buffer because its preparation is far simpler. The calculation of pH is also far easier, because the dissociation constants at various ionic strengths (19) and temperatures (20) have been worked out, while in the case of citrate the determination of pH always depends upon the experiments, because it has three dissociation constants, the changes of which with ionic strength are unknown (21).

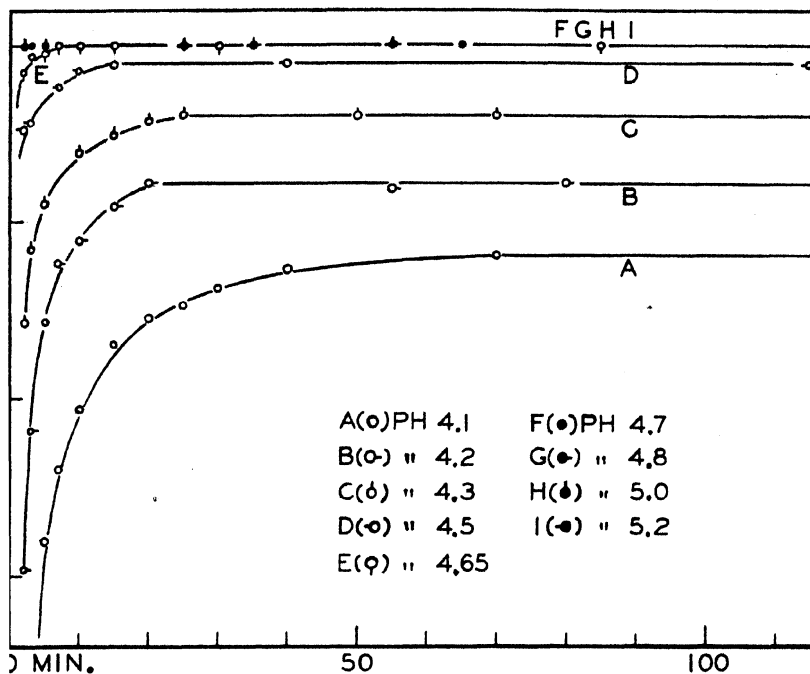


FIG. 1. Increase in color intensity ( $I$ ) with time of cysteine solution in media of different pH. Color standard,  $4 \times 10^{-4}$  M cysteine. Color mixture containing 0.4 M sodium acetate, varying concentrations of acetic acid,  $4 \times 10^{-4}$  M cysteine,  $8 \times 10^{-3}$  M HCl, and 4 cc. per 50 cc. total volume of phospho-18-tungstic acid reagent.

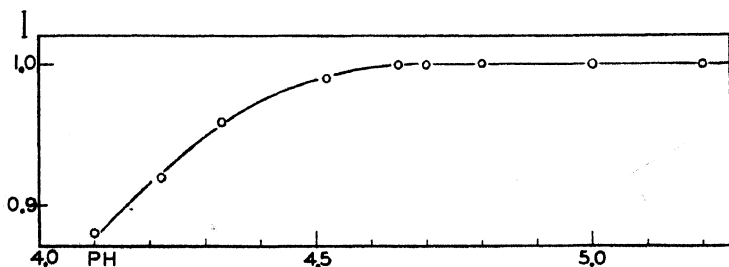


FIG. 2. Maximum color intensities ( $I$ ) developed by  $4 \times 10^{-4}$  M cysteine at various pH. Color standard,  $4 \times 10^{-4}$  M cysteine.



citrate and sodium bicarbonate buffer, it was found that this constancy of color is valid until the pH reaches about 8.0, beyond which there is a slight increase in intensity. However, the fading of the color becomes more rapid as pH increases.

For this reason, 10 cc. of 2 M sodium acetate solution and 3 cc. of 2 M acetic acid are used in the color standard and all the other experiments. Moreover, at this pH the colors of interfering substances are considerably minimized.

*Effect of Increasing Amount of Phospho-18-Tungstic Acid Reagent upon Color Intensity*—The kind and amounts of components of the reaction mixtures are the same as those of the color standard, except that the amount of the reagent is varied (2 to 15 cc.) with previous addition of NaOH to neutralize its acidity (pH 5.0). The color developed by greatly different amounts of the reagent is uniform within the experimental error inherent in the determination (Fig. 3). This phenomenon may be understood if the reaction between cysteine and the reagent proceeds to 100 per cent completion. A strict proportionality existing between the color intensity and an active substance of the reagent, in the presence of an excess of cysteine, makes the method useful for the quantitative determination of the active substance, phospho-18-tungstic acid.

*Effect of Dilution*—On dilution, either with water or with the acetate buffer, the color intensities of the final solutions are strictly proportional over the concentration range of  $4 \times 10^{-5}$  to  $4 \times 10^{-4}$  M cysteine. Such dilution may therefore safely be made, when necessary, for colorimetry.

*Cysteine Concentration and Color Intensity*—Proportionality<sup>8</sup> is also satisfactorily maintained on varying the amounts of cysteine taken, provided enough of the reagent is employed (Fig. 3). The minimum concentration of cysteine which gives noticeable color with the reagent under the specified conditions is  $4 \times 10^{-6}$  M, or

<sup>8</sup> Owing to errors occurring in colorimeter readings and measurements of volume, errors of  $-0.6$  to  $-6.0$  per cent below  $1.5 \times 10^{-4}$  M cysteine concentration and less than  $\pm 1.0$  per cent between  $2 \times 10^{-4}$  and  $16 \times 10^{-4}$  M were observed. If it is desired to decrease the error in the determination of cysteine at lower concentrations,  $4 \times 10^{-5}$  M cysteine solution may be used as the standard, with which cysteine from  $0.4 \times 10^{-5}$  to  $2.0 \times 10^{-4}$  M can be determined with errors of  $\pm 0.25$  to 5.0 per cent, which correspond to  $\pm 0.025$  to 0.5 per cent of the  $4 \times 10^{-4}$  M cysteine color standards.

0.482 mg. of cysteine per liter. The color can be determined with a total volume of 10 cc. instead of 50 cc., with very little more error. Therefore, the smallest amount of cysteine to be detected is 0.0048 mg.

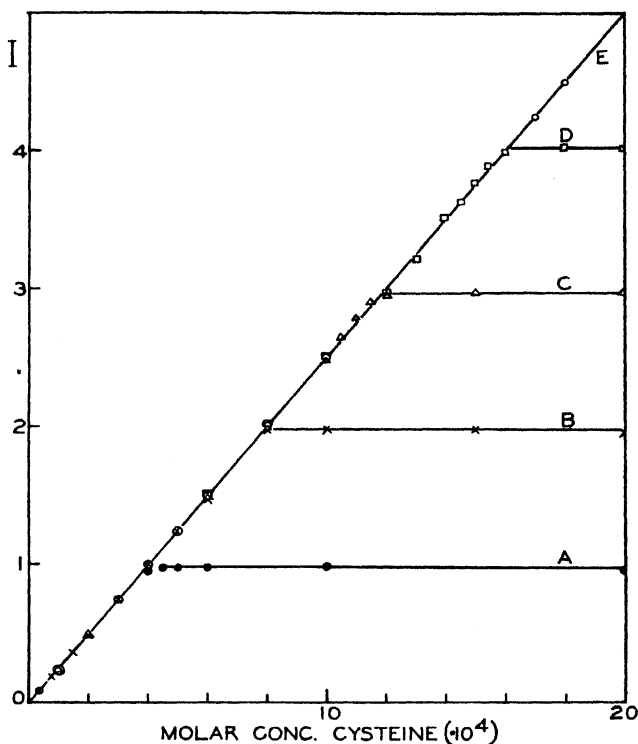


FIG. 3. Increase in color intensity ( $I$ ) against cysteine concentration. Color standard,  $4 \times 10^{-4}$  M cysteine. ● 20 cc. per liter of phospho-18-tungstic acid reagent (Curve A); × 40 cc. (Curve B); △ 60 cc. (Curve C); □ 800 cc. (Curve D); ○ 100 cc. (Curve E).

*Thioglycolic Acid Concentration and Color Intensity*—With various amounts of 0.01 M thioglycolic acid in 0.2 M HCl, the same experiments as in the case of cysteine were performed. The cysteine color standard was also used in this series of experiments.

The results showed proportionality of the color intensity with the thioglycolic acid concentration ( $\pm 2$  per cent), and also that the same number of moles of cysteine and thioglycolic acid produce the same color intensity. The rate of color development of the acid is also practically the same as that of cysteine.

*Other Thiol Compounds*<sup>9</sup>—Methionine was found to give no coloration even after 24 hours, as theoretically predicted for such a linkage as  $-\text{S}-\text{CH}_3$ .

0.01 M  $\text{Na}_2\text{S}$  solution was made from crystalline sodium sulfide (Merck's reagent grade).  $\text{Na}_2\text{S}$  develops color exponentially with time, but the rate is very slow. For instance, solutions containing  $6 \times 10^{-4}$  M and  $1 \times 10^{-3}$  M were found to produce respectively 0.18 and 0.33 unit of color in 10 minutes, when distinct turbidity appears, preventing colorimetry. The precipitate is very likely sulfur. The color slowly increases, even after the appearance of the precipitate and the maximum cannot be defined. It was proved that bubbling nitrogen gas, preferably under reduced pressure, through acid cysteine solution frees it from its  $\text{H}_2\text{S}$  content, and the resultant solution gives the same intensity as one containing no  $\text{H}_2\text{S}$  originally.

With *n*-butyl mercaptan the color develops with time exponentially and very slowly, as for  $\text{Na}_2\text{S}$ , and the color intensities developed are roughly the same as for the corresponding amount of  $\text{Na}_2\text{S}$  for about 10 minutes, after which the rate decreases greatly. Disulfide precipitation was observed in 7 to 10 minutes with a solution which contained more than  $4 \times 10^{-4}$  M. No proportionality between the color intensities and the mercaptan concentrations could be observed at any time, a solution containing less mercaptan sometimes showing greater intensity. The irregularities are, no doubt, due to its insolubility. Others, like ethyl mercaptan and thiocresol behave in a similar manner. These water-insoluble

<sup>9</sup> Glutathione was examined, the Eastman Kodak Company's preparation being used. Various peculiarities were observed, especially that, as its solution in 0.2 M HCl stands, the maximum color intensity increases until after 15 days it reaches 82.5 per cent of that produced by an equivalent amount of cysteine, beyond which there is no increase. This is probably due to the fact that the sample used was in the form of a disulfide, which hydrolyzes very rapidly compared to cysteine. This peculiarity will be studied when pure glutathione is obtained.

mercaptans were found to be easily extracted by shaking the solution with chloroform or with ether.

Cysteine solution mixed with the mercaptan showed the anticipated intensity after being shaken once with the same volume of chloroform.

*Effect of Salts upon Color Development*—By increasing the salt concentration, three effects are possible. The first is upon the pH of the medium due to the change in the classical dissociation constant of acetic acid; the second is that of the added salts upon thiol compounds; the third upon phospho-18-tungstic acid. All these three effects will influence the rate and the intensity of color development. The first effect is negligible because the change in ionic strength from 0.4 to 2.0 M shifts the pH of the medium only from 5.0 to 5.1, which has an imperceptible effect on the color. The second effect is brought about when salts of heavy metals like silver and mercury are added. They combine in the pH range with thiol compounds to form mercaptides, decreasing or destroying the color.<sup>10</sup> The third effect is caused by ions of zinc, alkali-earths, etc. They form precipitates with phospho-18-tungstic acid, preventing color production.

Even at a concentration as high as 1.2 M, LiCl, LiSO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, KNO<sub>3</sub>, and CCl<sub>3</sub>COONa have not the slightest effect upon either the rate or intensity of color production, although (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and Na<sub>2</sub>SO<sub>4</sub> at high concentrations (about 1.0 M) produce large square crystals after 40 hours, and KNO<sub>3</sub> at 0.4 M produces rhombic crystals in 20 hours, and at 0.8 M in 2 hours. CH<sub>3</sub>COONa also shows no influence as long as the medium is maintained in the neighborhood of the specified pH region. On the contrary, the halides of K, Na, NH<sub>4</sub>, and Mg show considerable influence which depends upon the length of time they are in contact with the reagent before cysteine is added; the longer they are in contact, the less color is developed. Thus, NH<sub>4</sub>Cl at 0.4 M produces 93 per cent color after contact of 1 minute, 71 per cent after 3 minutes, and 60.2 per cent after 5 minutes. NaCl at 0.4 M produces about 90 per cent after contact of 1 minute and less than 10 per cent after about 5 minutes. KCl at 0.4 M produces only 81 per cent after contact of 1 minute.

<sup>10</sup> This effect of Hg salt will be described in detail in a forthcoming paper.

If cysteine is added to the reaction medium before the reagent, which is the specified procedure, these salts below 0.4 M show practically no effect upon the maximum color intensity and but little effect upon its rate of development. However, they show marked effect beyond that concentration.

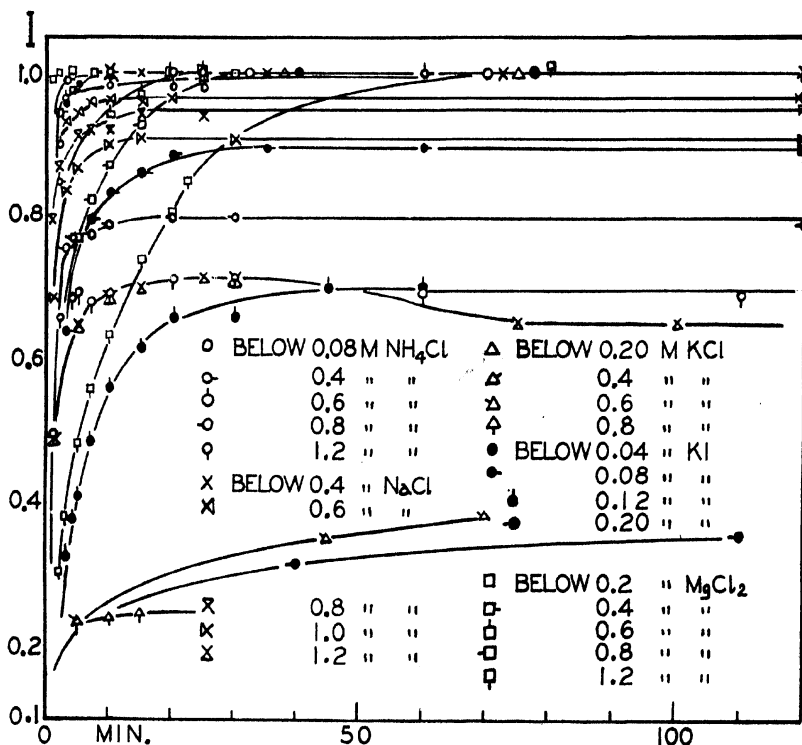


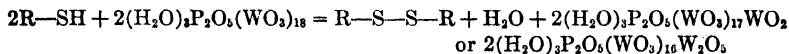
FIG. 4. Effect of halides upon the color production of cysteine and phospho-18-tungstic acid. Color standard,  $4 \times 10^{-4}$  M cysteine.

Among the halides examined, KI has the most prominent effect, even at 0.08 M greatly retarding the color development and diminishing by 10 per cent the maximum intensity. The effect becomes stronger as its concentration increases (Fig. 4) and beyond 0.2 M there is abundant formation of precipitate (minute colorless cubical and rhombic crystals) after 4 hours. KCl beyond 0.4 M shows a

distinct effect (Fig. 4). Even in a solution of 0.4 M precipitation of minute colorless cubical crystals was observed after 2 hours.  $MgCl_2$  slightly retards the color development at 0.4 M and more strongly as its concentration increases. However, it has no effect upon the maximum intensity (Fig. 4).  $NaCl$  beyond 0.6 M depresses the maximum intensity, although it has but slight effect upon the rate of its development (Fig. 4). No precipitate formation was observed even at 1.2 M for at least 6 hours.  $NH_4Cl$  also has only slight effect upon the color development, but depresses the maximum intensity by 2 per cent at 0.6 M, which decreases as its concentration increases (Fig. 4). Precipitation of large crystals was observed after 24 hours in solution containing more than 0.8 M  $NH_4Cl$ . Potassium and sodium cyanide have the greatest effect of all the salts examined. They inhibit color even at 0.0025 M for about 30 minutes, after which slight color appears.

#### DISCUSSION AND SUMMARY

Wu's study (22) leaves no doubt that the reduction of phospho-18-tungstic acid reagent by thiol compounds causes the color production. As seen in Fig. 4, the minimum amount of cysteine that produces maximum color intensity with 1 cc. of phospho-18-tungstic acid is approximately  $2 \times 10^{-4}$  moles. 1 cc. of the reagent contains roughly  $3.5 \times 10^{-4}$  moles of  $WO_3$ , which corresponds to  $1.94 \times 10^{-5}$  moles of phospho-18-tungstic acid. Therefore, it would be concluded that cysteine reacts with the complex acid with 1:1 ratio. Moreover, it was proved by Lugg (9) and also will be shown by the author in another paper, that the oxidation of the cysteine in this reaction does not proceed beyond the cystine stage. Thus the reaction between the complex acid and cysteine or thioglycolic acid may be expressed by the following stoichiometrical equation:



Advantage of the use of acetate buffer (pH 5.0) as the reaction mixture was pointed out.

Adjustment of the pH of the reagent was not made in this series of experiments, but was found possible, without destroying its

composition, by means of LiOH. According to Wu (22), a pure A-phospho-18-tungstic acid can be prepared. Though important for chemical studies, it is not for the purpose of thiol compound determination, provided its relation to cysteine is known.

The oxidation-reduction equilibrium of the complex acid and its reduced form has not been studied so far, although it is important for the elucidation of the color reaction. Indifference of cystine to the color suggests that the reaction between the complex acid and a thiol compound is the shift of the oxidation-reduction equilibrium of the former compound by the apparently irreversible oxidation of cysteine. The relation between the rate of color development by thiol compounds and the pH indicates that reduction of the complex acid is due to the ionized thiol group.

The very slow color production by the insoluble thiol compound is, no doubt, due to its insolubility, and the rate is probably that of diffusion. From the analytical standpoint, such insoluble mercaptans can be easily extracted with ether or preferably with chloroform, and thus present no special difficulty.

H<sub>2</sub>S, which may be present mostly as a decomposition product in the solution for which cysteine (or other thiol compound) is to be determined, was shown to be easily removed by means of nitrogen or carbon dioxide gas. The indifference of the disulfides, uric acid, and amino acids makes the method very useful.

Owing to their retarding effect upon the color development, the use of halides at a high concentration in any stage of thiol determination should be avoided. Thus, for the hydrolysis of proteins, H<sub>2</sub>SO<sub>4</sub> should replace HCl, and, if the latter is used for some unavoidable reason, it should be neutralized with LiOH.

The effect of the phosphate buffer upon the complex acid is apparently due to the K ion present in the form of potassium phosphate. It was shown that potassium and ammonium salts precipitate the reagent.

The predominant effect of cyanide is unknown. Wu used sodium cyanide in a concentration of about 0.02 M in the colorimetric determination of uric acid, apparently without any noticeable peculiarity, while in the present experiment even 0.0025 M NaCN was shown to inhibit the color development for some time. The difference is probably due to the difference in pH of the two media, the former being in the range of NaCO<sub>3</sub>, while the latter is

5.0. Owing to the color-retarding effect of KCN, it cannot replace sulfite, which is used in the determination of cystine.

In the light of the experimental results, the procedure described under "Technique" is justified as a general analytical method for cysteine and thioglycolic acid.

The author would like to express his gratitude to Mr. Robert McNeil, Dr. Stanley P. Reimann, and Dr. Frederick S. Hammett for their assistance in various ways. He also would like to thank Dr. Vincent du Vigneaud for his kind gift of the methionine used in the experiment.

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## THE ALKALOIDS OF HAN-FANG-CHI

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The Chinese drug fang-chi probably includes several species of menispermaceous plants. One variety that is commonly sold in Chinese drug stores is known as han-fang-chi apparently because it was originally cultivated in Han Chung (1). There is considerable uncertainty concerning the botanical identification of han-fang-chi. Hoffmann and Schultes (2) gave it the name *Cocculus japonicus*, while "Botanical nomenclature" (3) classified it as *Cocculus diversifolius*. The last designation may not be correct because Ohta (4) reported the isolation of kukoline,  $C_{16}H_{20}O_3N \cdot 3H_2O$ , m.p.  $162^\circ$ , and diversine,  $C_{16}H_{20}O_4N$ , m.p.  $144-154^\circ$ , from *Cocculus diversifolius*, while the alkaloid present in han-fang-chi, as described below, is obviously different from either of these.

The part of the plant that is employed in Chinese medicine is the root. Han-fang-chi has been advocated as a diuretic, an expectorant, and a cathartic (1). It has also been recommended for the control of rheumatic pain and hemoptysis.

A preliminary study of han-fang-chi was undertaken by Kubota (5) who separated an alkaloid which had a melting point of  $217^\circ$ , specific rotation  $[\alpha]_D^{18} = +262.8^\circ$ , and an empirical formula  $C_{19}H_{23}O_3N$ . He believed that his alkaloid was identical with tetrandrine, which Kondo and Yano (6) isolated from the Japanese plant *Stephania tetrandra*.

In 1931 a shipment of han-fang-chi was made available to us from Shanghai. These roots on the average measured 1.6 cm. in diameter. They were already cut in short pieces, 3.2 cm. long, and were split in the center longitudinally. The covering of the root is light brown, while the cut surface appears pale white.

Our specimen was subjected to alkaloidal assay and ash analysis.<sup>1</sup> As shown in Table I, han-fang-chi has an alkaloidal content of 2.3 per cent, the U.S.P. assay process for belladonna leaves being used (7). It gives off an average of 9.09 per cent of moisture and volatile matter when heated to 100°, and yields 3.48 per cent of ash upon incineration. The ash is more soluble in 10 per cent hydrochloric acid than in water. Iron and aluminum oxides are present in the ash to the extent of 25.03, calcium 14.01, phosphorus 5.02, magnesium 4.85, and sulfur 2.18 per cent. Both potassium and sodium were detected.

TABLE I  
*Analysis of Han-Fang-Chi*

Analysis for	Sample 1	Sample 2	Sample 3	Average
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Total alkaloids.....	2.40	2.21		2.30
Moisture and volatile matter.....	9.15	9.08	9.03	9.09
Total ash.....	3.47	3.49	3.48	3.48
Water-soluble ash.....	43.66	45.04	44.63	44.44
HCl-soluble ash.....	48.07	47.33	46.37	47.26
Insoluble ash.....	8.27	7.63	9.00	8.30
Mg in ash.....	4.87	4.85	4.82	4.85
S " ".....	2.25		2.10	2.18
Fe <sub>2</sub> O <sub>3</sub> and Al <sub>2</sub> O <sub>3</sub> in ash.....	25.09		24.98	25.03
P in ash.....	4.97	5.02	5.07	5.02
Ca " ".....	13.89		14.12	14.01

The chief interest of our investigation was, of course, in the isolation of alkaloids. For this purpose, 11 kilos of the powdered drug were percolated with 95 per cent alcohol until the alkaloids were practically exhausted. The alcoholic extract was distilled under diminished pressure to a very small volume. The thick syrup was dissolved in 1 per cent hydrochloric acid. To the acid solution, which was filtered, a 1 per cent solution of sodium hydroxide was added, and the heavy precipitate thus formed was separated by suction. Most of the precipitate was soluble in warm acetone and gave rise to a uniformly crystalline body. The

<sup>1</sup> We are indebted to Mr. Walter Hoover for his alkaloidal assay and to Mr. Robert C. Anderson for his ash analysis.

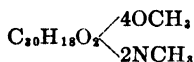
substance, undoubtedly a base or an alkaloid, was finally purified by recrystallization. It forms fine, colorless needles and is soluble in acetone, chloroform, and ether, less soluble in ethyl alcohol, but insoluble in water and petroleum ether. It exhibits a slight green fluorescence in acetone solution. When dissolved in a dilute acid solution, it reacts with most of the alkaloidal reagents. It melts at 217–218° (corrected) and has a specific rotation  $[\alpha]_D^{20} = +252.4^\circ$  in chloroform.

The results of our combustion analyses and molecular weight determinations<sup>2</sup> agree with those of Kondo and Yano (8) for tetrandrine, and, provisionally, we therefore also adopt their revised empirical formula  $C_{38}H_{42}O_6N_2$ .

*Analysis*— $C_{38}H_{42}O_6N_2$

Calculated.	C 73.26,	H 6.80,	N 4.50,	mol. wt. 622
Found.	" 73.00,	" 6.96,	" 4.43,	" " 559
	" 73.00,	" 6.80,	" 4.39,	" " 549
			" "	565

It should be noted that the observed molecular weight is lower than the theoretical value, although it closely agrees with Kondo and Yano's (8) figures (572, 552, 530). It differs from their original data (309, 298) as well as Kubota's (5) determination (309). The substance apparently contains four methoxy and two methylamino groups as shown by the following analyses.



Calculated.	4OCH <sub>3</sub> 19.94,	2NCH <sub>3</sub> 4.83
Found.	" 19.63, 18.47, 19.31,	" 4.16, 4.44, 4.99

Although the alkaloid is easily crystallized, the crystallization of its salts offers much more difficulty. We were successful in preparing a series of six new salts. They may be briefly described as follows:

*Tetrandrine hydrochloride*,  $C_{38}H_{42}O_6N_2 \cdot 2HCl$ , can be best prepared by suspending the pure base in a small amount of water and adding, drop by drop, 5 per cent hydrochloric acid until the solu-

<sup>2</sup> The analyses reported in this paper were made by Dr. Ing. A. Schoeller, Berlin-Schmargendorf, Germany, but the molecular weight determinations were made by Dr. A. M. VanArendonk of our own department according to the Rast method.

tion is just acid to litmus. The whole is evaporated to dryness and redissolved in absolute alcohol or a mixture of butyl and ethyl alcohol. The hydrochloride crystallizes in colorless prisms, softens at  $263^{\circ}$  (corrected), and foams at  $266^{\circ}$  (corrected). Its specific rotation is  $[\alpha]_D^{27} = +224.2^{\circ}$  in water. It is soluble in water and alcohol, less soluble in butyl alcohol, slightly soluble in acetone and chloroform, but insoluble in ether and petroleum ether.

*Analysis*— $C_{38}H_{42}O_6N_2 \cdot 2HCl$ . Calculated, Cl 10.49; found, Cl 10.52.

*Tetrandrine hydrobromide*,  $C_{38}H_{42}O_6N_2 \cdot 2HBr$ , can be made and purified in a similar manner to the hydrochloride. The salt forms short colorless needles, and has a specific rotation of  $[\alpha]_D^{27} = +200.7^{\circ}$  in water. It colors at  $258^{\circ}$ , softens at  $268$ – $278^{\circ}$ , and foams at  $270^{\circ}$  (corrected). Its solubility is practically the same as that of the hydrochloride.

*Tetrandrine nitrate*,  $C_{38}H_{42}O_6N_2 \cdot 2HNO_3$ , may be prepared by dissolving the alkaloid in 50 per cent alcohol with 5 per cent nitric acid. Purification should be carried out in 95 per cent alcohol. The nitrate crystallizes in prisms, single or in rosettes, and has a specific rotation of  $[\alpha]_D^{29} = +211.2^{\circ}$  in water. It softens at  $205^{\circ}$  and decomposes at  $208^{\circ}$  (corrected). It is soluble in water and alcohol, but insoluble in ether, petroleum ether, benzene, acetone, and chloroform.

*Tetrandrine oxalate*,  $C_{38}H_{42}O_6N_2 \cdot H_2C_2O_4$ , may be obtained by the same method as the hydrobromide. It crystallizes in white needles, softens at  $147.5$ – $148.5^{\circ}$ , and foams at  $165$ – $170^{\circ}$  (corrected).

*Tetrandrine picrate*,  $C_{38}H_{42}O_6N_2 \cdot 2C_6H_3O_7N_3$ , is formed when a saturated solution of picric acid is dropped into an acetone solution of tetrandrine. The insoluble yellow salt is filtered off and thoroughly washed with water, alcohol, and ether. It decomposes at  $235$ – $242^{\circ}$  (corrected).

*Tetrandrine flavianate*,  $C_{38}H_{42}O_6N_2 \cdot 2C_{10}H_6O_8N_2S$ , may be prepared in a similar manner to the picrate. It is yellow in color and insoluble in most solvents. It decomposes at  $248$ – $250^{\circ}$  (corrected).

There is evidence that han-fang-chi contains other alkaloids in addition to tetrandrine. This is now being further investigated. The pharmacological results on tetrandrine will be presented in another communication.

## SUMMARY

The Chinese drug han-fang-chi contains an average of 2.3 per cent of total alkaloids.

An alkaloid has been isolated in pure form. The analytical data conform to the empirical formula of tetrandrine,  $C_{38}H_{42}O_6N_2$ . It has four methoxy and two methylamino groups. A series of six new salts has been prepared—the hydrochloride, the hydrobromide, the nitrate, the oxalate, the picrate, and the flavianate.

Upon ignition, han-fang-chi yields 3.48 per cent ash. The elements present in the ash are iron, aluminum, calcium, potassium, sodium, sulfur, and phosphorus.

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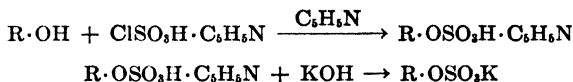
# A NEW METHOD FOR THE SEPARATION OF STEROLS FROM VITAMIN D-CONTAINING MATERIALS

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(Received for publication, January 28, 1935)

In an earlier study (1) it was found that the inorganic salts of sterol sulfates were extremely insoluble in most organic solvents and for most salts practically insoluble in cold water. The sterols are readily transformed to these salts by means of pyridine chloro-sulfonate in the presence of excess pyridine, followed by treatment with potassium hydroxide. The reaction has since been found to be quantitative<sup>1</sup> and may be represented by the following equations where R represents a sterol residue.



The salt formed is insoluble in organic solvents. It can be extracted in a continuous extractor with diethyl ether or petroleum ether without a measurable loss of weight.

Sterols are found widely distributed in nature. It is intended to apply this reaction for their separation and determination. For the isolation of large amounts of material it has the advantage over nitrated benzoyl chloride in that it is quantitative, the derivative formed being far less soluble in organic solvents than the dinitrobenzoate esters. Quantitatively, it has advantages over digitonin in that it is easier to handle, less expensive, and offers a compound of definite composition which may be analyzed for sterol, potassium, or sulfate.

<sup>1</sup> By the use of this method 0.1 mg. of cholesterol was recovered quantitatively within the limits of error of the Liebermann-Burchard reaction. Details will be reported later in connection with the applicability of this method to blood and other biological materials.



The reaction was applied to cod liver oil concentrate. It was expected that the antirachitic vitamin, apparently sterol-like in structure, would form an insoluble sulfate derivative and so be found with the sterol fraction, from which it could be separated by digitonin. This would afford a simple method for high concentration of the natural vitamin.

The vitamin did not form an insoluble sulfate derivative. The insoluble fraction proved to be a mixture of sterol sulfates having no antirachitic action. All the antirachitic activity, within experimental error, was accounted for in the petroleum ether-soluble fraction which was about two-thirds the weight of the original non-saponifiable fraction. This fraction also gave the typical color test for vitamin A with antimony trichloride in chloroform solution, while the insoluble fraction gave no such test.

Evidence is rapidly accumulating that the antirachitic vitamin from different sources may not be the same entity (2-6). Therefore, the behavior of irradiated ergosterol and cholesterol toward this reagent was studied. In both cases, two fractions were separated as in the case of cod liver oil concentrate. Again, the insoluble sterol sulfate fractions were inactive antirachitically, whereas the ether extracts were highly active.

In all the above cases the objection might be raised that so small a concentration of the vitamin is present that conditions for isolation of its derivative are unfavorable. The reaction was applied, therefore, to a high concentrate of the vitamin, calciferol (40,000 international units per mg.). In this case, only a petroleum ether-soluble fraction was isolated, which retained all the antirachitic activity of the original material. On ashing, an inorganic residue did not remain, which eliminated the possible objection that a salt might form which was soluble in petroleum ether.

From these experiments it is evident that the antirachitic vitamin is inert to pyridine chlorosulfonate. This reagent can be used, therefore, in the separation of ergosterol and cholesterol from the vitamin.

In view of the above observations, the limitations of this reagent are of importance. Since derivatives of phenol (7), ergosterol (1), and cholesterol (8) are known, it was of interest to note whether a cyclic compound containing a more typically alcoholic hydroxy group, such as borneol, would react with this reagent. Borneol

was successfully made to react to form a potassium bornyl sulfate. Although further work is necessary in this direction before a significance can be attached to these observations, it is apparent that this reagent reacts with hydroxy groups attached to cyclic nuclei widely differing in nature, but not with the antirachitic vitamin.

It is apparent that on irradiation to form antirachitic substances some change takes place so that a potassium sulfate derivative cannot be obtained. In view of these experiments an explanation is necessary for the observed activation of ergosterol sulfate in aqueous medium (1). Accordingly dry potassium ergosterol sulfate was irradiated and found to be inactive. This same material, after irradiation in the dry state, was then boiled with water in order to see if the substance had been activated and was prevented from healing rickets because of the inhibition of the sulfate radical on antirachitic utilization. Again the material was found to be inactive. Boiling with water first and then irradiating, however, rendered the material powerfully active, indicating that no change had taken place in the ergosterol residue. These experiments harmonize the inactivity of the vitamin to pyridine chlorosulfonate and the activation of ergosteryl sulfate in aqueous medium.

The described method for the separation of sterols is being applied to the determination of sterols in biological materials.

#### *General Method for Separation of Sterols from Vitamin-Containing Materials*

Pyridine chlorosulfonate was prepared by adding chlorosulfonic acid to a cooled solution of pyridine in chloroform. For each gm. of sterol (dissolved or suspended in 5 cc. of chloroform) 0.4 cc. of chlorosulfonic acid was added to 3 cc. of pyridine dissolved in 5 cc. of chloroform. Pyridine chlorosulfonate crystals separate out. However, this does not hinder the reaction in any way. To this salt suspension in chloroform and excess pyridine is added the solution of sterol. The mixture is allowed to stand for a few minutes with occasional shaking and then refluxed gently for 2 hours. The pyridine and chloroform are evaporated off under a vacuum and 1 gm. of KOH dissolved in 10 cc. of water is added and the mixture cooled with crushed ice. Vigorous shaking is necessary to effect complete transference from the pyridine to the potassium salt. In most cases the salt may be easily filtered off

with suction on a Buchner funnel and washed with water, alcohol, and then diethyl ether. In the case in which a large amount of oily material is present, as in the case of cod liver oil concentrate, the material may be centrifuged from the water, and washed then with alcohol and diethyl ether. The residue is then continually extracted in a continuous extractor with anhydrous diethyl ether, dried, and weighed.

The combined extracts are evaporated under a vacuum at 35° so that almost all the alcohol and ether are removed, leaving a mixture of water and oil. The mixture is then extracted several times with petroleum ether, the petroleum ether being washed several times with water to remove as much pyridine as possible, dried over anhydrous sodium sulfate, and then evaporated off under a vacuum. For large quantities of sterol, the amount of solvent may be decreased to about a total of 500 cc. of chloroform in a reaction with 100 gm. of material.

The course of the reactions, amounts used, and yields may be found in Table I.

*Cod Liver Oil Concentrate*—17 gm. of the vitamin concentrate (10,000 international units per gm. prepared according to Marcus (9)) were treated as in the general method, calculated on the basis of two-thirds of the material being sterol. The white crystalline sulfate salt obtained (5.9 gm.), recrystallized from large amounts of methyl alcohol, contained 7.24 per cent potassium, gave the blue-green color characteristic of cholesterol with chloroform, acetic anhydride, and sulfuric acid, and decomposed on rapid heating at 235–242°. This substance showed no antirachitic activity when administered in large doses and could only be activated if it were first boiled with water and then irradiated. Even then large doses (100 mg.) were needed for efficient healing.

The petroleum ether extract was a heavy, semisolid, transparent material (11.1 gm.). 2 mg. of this oil produced a degree of healing in rachitic rats in 5 days which was equivalent to approximately 3 mg. of the starting material. The vitamin concentrate gave a transient blue, rapidly changing to a pink and then a light brown with the chloroform, sulfuric acid, and acetic anhydride reagent.

*Irradiated Ergosterol*—3 gm. of ergosterol, dried in a desiccator, were irradiated with a mercury vapor lamp at a distance of 1 foot for 30 minutes. This was treated as in the general method. The

TABLE I  
*Fractionation of Vitamin-Containing Materials*

Cod liver oil concentrate (17 gm.)	Ergosterol (3 gm.); irradiated	Cholesterol (10 gm.); irradiated
Treated with chlorosulfonic acid reagent	Treated with chlorosulfonic acid reagent	Petroleum ether-extracted (250 cc.)
Sterol sulfate (5.9 gm.); inactive	Sterol sulfate (3.2 gm.); inactive	Treated with chlorosulfonic acid reagent
Petroleum ether-soluble fraction (11.1 gm.); active	Petroleum ether-soluble fraction (0.30 gm.); active	Sterol sulfate (3.4 gm.); inactive
		Petroleum ether-soluble fraction (0.23 gm.); active
Boiled with water, irradiated; inactive	Boiled with water, then irradiated; active	Boiled with water, irradiated; inactive
↑	↑	↑
Boiled with water after irradiation; inactive	Boiled with water after irradiation; inactive	Boiled with water after irradiation; inactive

ergosterol sulfate obtained (3.2 gm.) was inactive antirachitically. This salt was inactive on irradiation, nor could it be activated when the irradiated salt was boiled in water. Boiling 10 micrograms of this salt in water for a few minutes and then irradiating for 3 minutes at a distance of 6 inches from the lamp produced excellent healing in rachitic rats in 5 days.

The petroleum ether extract (0.3 gm.) was a yellow semisolid, viscous, transparent material, interspersed with occasional crystals. 3 micrograms of this material would produce excellent healing in rachitic rats in 5 days. A more quantitative study of the potency of this material is to be made.

*Irradiated Cholesterol*—10 gm. of dry cholesterol were irradiated for 1 hour, as in the case of ergosterol. The irradiated material was then extracted with 250 cc. of petroleum ether. The petroleum ether was evaporated off and the residue (3 gm.) was treated as in the general method. 3.4 gm. of sterol sulfate and 0.23 gm. of cholesterol vitamin concentrate were obtained on extraction with 250 cc. of petroleum ether. The sulfate was inactive, while the petroleum ether fraction contained all the activity. The soluble heavy oil gave a weak sterol test with the chloroform-sulfuric acid-acetic anhydride reagent, giving a faint green, different in appearance from that produced by a similar amount of cholesterol.

*Calciferol*—0.5 gm. of calciferol (experiment done in duplicate) was treated as in the general method. On addition of the KOH solution an oil separated and was recovered by extraction with petroleum ether. No petroleum ether-insoluble salt was obtained. On evaporation of the petroleum ether the calciferol crystallized and its potency was checked against the original material by feeding 0.34 microgram of each material to rachitic rats. The extent of healing was compared after 5 days. No measurable loss of potency was observed. A small sample of the petroleum ether extract was ashed, but left no residue.

*Bornyl Sulfate*—This material was prepared after the general method. 7.7 gm. of borneol yielded 13 gm. of shiny, thin plates of potassium bornyl sulfate.  $C_{10}H_{17}OSO_3K$ , K (calculated) 14.34, K (found) 14.26, decomposed at  $220^\circ$ . 1 gm. dissolves in 20 cc. of water. The borneol may be recovered by steam distillation.

*Biological Technique*—Albino rats raised in our laboratory from an original Wistar strain were used. The mothers were kept on a

Bills (10) stock diet. The young were weaned at 21 days. At this time they were placed on the stock diet. At 23 to 25 days administration of the experimental diet was begun. This was a modified Steenbock-Black (11) rickets-producing diet of the following composition: corn-meal 70, wheat gluten 16, brewers' yeast 10,  $\text{CaCO}_3$  3,  $\text{NaCl}$  1 per cent.  $\text{Ca} = 1.2$ ,  $\text{P} = 0.24$  per cent. This diet, developed in our laboratory, has proved to be highly satisfactory. The animals, while developing marked rickets, grow better than they do on other rickets-producing diets.

After a period of about 18 days, when the Roentgenograms showed evidences of marked rickets, the animals were given the material to be tested in 0.1 cc. of maize oil. The controls were given only the maize oil. In a few cases a 0.1 cc. water suspension was used. At the end of 5 days the degree of healing was determined Roentgenographically and by the  $\text{AgNO}_3$  stain on the tibiae. In most cases, semiquantitative results were desired and hence two rats were used for each test, controlled by two other animals. None of the controls, during all these experiments, showed spontaneous healing. Most of the experiments were carried out at least twice. In some cases, where an approximate assay was desired, the experiments were repeated three times. The degree of healing was interpreted by means of standardized solutions of viosterol. Testing by a single dose, used in this investigation, was recommended recently by Coward and Key (12).

#### SUMMARY

1. Sterols may be separated efficiently from vitamin D-containing material such as cod liver oil, irradiated cholesterol, and irradiated ergosterol by converting them to potassium salts of their sulfuric acid esters.

2. Dry potassium ergosterol sulfate cannot be activated antirachitically. It may be activated after boiling in aqueous medium.

3. Insoluble potassium sulfate derivatives may be isolated from phenol, ergosterol, cholesterol, and borneol when treated with pyridine chlorosulfonate in chloroform solution but not from the antirachitic vitamin in cod liver oil, calciferol, irradiated ergosterol, and irradiated cholesterol.

The authors wish to make the following acknowledgments: Calciferol was standardized and supplied by Dr. C. E. Bills of

Mead Johnson and Company. Ergosterol, brewers' yeast, and standardized viosterol were supplied by Mead Johnson and Company. After the first experiments the cod liver oil concentrate was supplied by the International Vitamin Corporation. The low phosphorus corn-meal was supplied by the Quaker Oats Company. Mr. Ronald Ellis helped in the breeding and care of the animals.

The authors are indebted to Dr. Benjamin Kramer for his valuable suggestions during this study.

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## **GALACTOSE IN THE THORACIC LYMPH OF THE DOG\***

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(Received for publication, February 12, 1935)

Absorption of readily diffusible substances by way of the lymph seems probable and in the case of levulose (1) and of amino acids (2) it has been shown to occur. Search of the literature showed no figures for the galactose content of thoracic lymph after the administration of that sugar. The presence of galactose in the portal vein has been demonstrated and it seemed of interest to determine whether or not galactose was absorbed to any extent in the lymph and if so in what quantity.

The work of Markowitz and Mann (3) demonstrated that ligation of the peripheral lymphatics or even complete hepatectomy did not affect the flow of lymph from the thoracic duct. Higgins and Lemon (4) and Higgins and Graham (5) investigated the lymph system in the dog. They concluded that all of the material absorbed into the alimentary lacteals appears finally in the thoracic duct and that this vessel plays an insignificant rôle in the drainage of lymph from visceral parts other than the intestine. We felt justified therefore in regarding thoracic lymph as truly representative of the material drained from the intestinal lacteals.

The effect of ingestion of galactose on blood glucose has been studied by Corley (6) and by Cori and Cori (7) who reported no increase of blood glucose after intravenous or intestinal administration of galactose. Conflicting results have been recorded by others (8-11), so this point seemed worthy of reinvestigation.

\* The data in this paper have been taken from a thesis presented by Mr. Wharton to the Graduate School of the University of Texas in partial fulfillment of the requirements for the degree of Master of Arts.



*Methods*

Dogs were used as experimental animals. 18 to 24 hours before the beginning of the experiment they were fed about a half pound of ground meat and a pint of whole milk. After the animal had been anesthetized by intraperitoneal injection of sodium amytal, the thoracic duct was exposed and a cannula introduced and one of the femoral veins laid bare. A blood sample was taken from the femoral vein and lymph collection was begun. The abdomen was opened and, after a normal lymph sample had been collected, a solution of galactose was injected into the duodenum. In making the injection the needle was pointed towards the pylorus in the first two animals, Dogs 25 and 26, but in the remaining experiments care was taken to direct the needle away from the pylorus. The syringe and needle were carefully removed and washed and the washings analyzed for galactose and this figure applied as a correction in determining the exact amount of sugar injected.

Blood and lymph were taken at definite intervals following the introduction of the sugar and were analyzed for total reducing sugar and for non-fermentable sugar by Somogyi's modification of the Shaffer-Hartmann method (12). The reducing value of galactose was found to be 70 per cent of the true value for Somogyi's Reagent 2 when tested with galactose solutions. The figures obtained with this reagent were multiplied, therefore, by the factor 1.43 to calculate the galactose content. A determination of the non-fermentable reducing substances ordinarily present in lymph was made with the Folin-Wu method of precipitation. Table I gives the results. Since the Somogyi precipitation satisfactorily eliminates this fraction, it was used throughout the experiments.

In addition to the femoral blood, a sample of mesenteric blood was taken just before terminating the experiment. It was taken at this time to avoid interrupting the normal circulation by manipulation and bleeding. At the end of the experiment, the animal was killed by injecting amytal directly into the heart. The alimentary tract was immediately removed and the galactose in the contents determined. In this way it was possible to determine the amount of galactose in the blood and lymph while

measuring the amount and rate of absorption of this sugar from the alimentary tract. The results are given in Tables I to III.

TABLE I

*Non-Fermentable Reducing Substances in Blood and Thoracic Lymph Determined on Folin-Wu Filtrates*

The reducing substances are measured in mg. per cent.

Blood					Lymph			
Dog No.	Source	Reducing substances as glucose			Dog No.	Reducing substances as glucose		
		Total	Non-fermentable	Fermentable		Total	Non-fermentable	Fermentable
1	Heart	89.0	11.2	77.8	5	103.0	11.0	92.0
	"	90.0	11.7	78.3		105.0	11.0	94.0
	"	85.5	9.0	76.5		151.0	12.0	139.4
	"	87.0	10.0	77.0		100.0	12.0	88.0
	"	88.5	11.0	77.5		112.0	12.0	100.0
2	"	85.0	9.0	76.0	6	117.0	7.5	109.5
	"	91.5	10.0	81.5		117.0	8.0	109.0
	"	90.0	8.0	82.0		115.0	7.5	107.5
	"	90.0	8.5	81.5		115.0	7.5	107.5
	"	90.0	8.5	81.5		123.0	7.5	115.5
3	Mesenteric vein	98.0	11.8	86.2	7	123.0	10.5	111.5
	Carotid artery	113.5	11.8	101.7		123.0	10.0	113.0
	" "	114.0	12.5	101.5		120.0	10.0	110.0
4	Mesenteric vein	98.0	11.8	86.2		106.0	5.0	101.0
	" "	111.5	11.0	100.5		106.0	7.0	99.0
	" "	111.5	10.0	101.5		118.0	9.0	109.0
						118.0	8.0	110.0
						103.5	7.5	96.0
						112.0	10.0	102.0
Average.....			10.3			9.1		

All sugar determinations were run in duplicate with Somogyi Reagent 2.

### Results

The results in Table II indicate clearly that galactose appears in the blood and thoracic lymph following the absorption of this sugar from the alimentary tract. It is also evident that there is a relationship between the rate of absorption of the sugar and the

extent to which galactose appears in the blood and lymph. In the two cases in which absorption was very slow, Dogs 25 and 26, galactose did not appear in the lymph or femoral blood in sufficient quantity to be detected by the analytical methods used. Mesenteric blood samples from these same dogs did show small but definite amounts of galactose. It will be recalled that in these two animals the injection of galactose was made with the needle directed towards the pylorus and very little absorption

TABLE II  
*Galactose in Thoracic Lymph and in Blood*

Dog No.	Rate of lymph flow	Absorption of galactose	Galactose absorbed by thoracic lymph in per cent of total absorbed	Maximum concentration of galactose			Galactose in urine*
				Femoral blood	Mesenteric blood	Thoracic lymph	
	cc. per min.	gm. per kg. per hr.		mg. per cent	mg. per cent	mg. per cent	
25†	0.44	0.29	0.00	0.00	10.7	0.0	—
26†	0.21	0.16	0.00	0.00	14.3	0.0	—
27	0.48	0.46	0.35	82.0	45.6	124.0	+
28	0.38	0.40	0.09	32.1	21.4	42.0	+
29	0.24	0.35	0.08	25.0	30.0	35.7	+
30	0.38	0.37	0.19	54.2	77.0	91.5	+
31	0.42	0.52	0.27	78.5	65.7	143.0	+
32	0.57	0.56	0.49	107.8	111.3	175.0	+
33‡	0.45		0.39	72.0	0.0	127.0	+
34‡	0.20		0.13	62.8	0.0	104.3	

\* Benedict's qualitative test for reducing sugar was performed on bladder urine at autopsy.

† Galactose injection made with the needle pointing towards the pylorus.

‡ The experiment was continued so long that calculation for absorption in gm. per kilo per hour was meaningless.

occurred. In the eight remaining dogs, galactose was found to appear in the blood, in the lymph, and in the urine. The quantity of galactose absorbed by way of the thoracic duct was found to be only a small part of the total absorbed. This is not surprising since the rate of flow of blood is so much greater than that of lymph. The figures in Table II show that the per cent of galactose absorbed through the thoracic duct increases with a greater flow of lymph.

Table III shows the effect of the galactose injection on the sugar content of blood and lymph. In all cases no galactose was found present in the preliminary blood sample, but in two, Dogs 33 and 34, small amounts of non-fermentable sugar were found present in the first lymph sample. In six of the nine animals an increase in blood glucose took place an hour after the injection, varying from 2.5 to 64.5 mg. One dog, No. 27, showed little change and two showed a decrease of 3.5 and 10.5 mg., respectively. In seven experiments there was an increase of glucose in the thoracic lymph, and in two there was a decrease. The general

TABLE III

*Effect of Galactose Injections on Sugar Level in Blood and Lymph*

The values are given in mg. per cent.

Dog No.	Blood glucose before giving galactose	Blood sugar 1 hr. after galactose		Lymph glucose before giving galactose	Lymph sugar 1 hr. after galactose		Change in glucose level	
		Glucose	Galactose		Glucose	Galactose	Femoral blood	Thoracic lymph
25	78.0	91.5	0.0	119.0	129.0	0.0	+13.5	+10.0
26	69.0	82.0	0.0	102.5	117.0	0.0	+13.0	+14.5
27	107.0	108.0	82.0	120.0	142.0	108.5	+1.0	+22.0
28	71.0	104.5	32.1	102.5	143.0	35.7	+37.0	+40.5
29	91.5	94.0	25.0	126.0	131.0	35.7	+2.5	+5.0
30	91.5	87.0	54.2	126.0	129.0	75.7	-3.5	+14.0
32	87.0	89.5	107.8	98.0	66.0	154.0	+2.5	-32.0
33	87.0	151.5	50.5	126.0*	185.0	117.0	+64.5	+59.0
34	102.5	92.0	44.0	130.0†	105.0	94.4	-10.5	-25.0

\* 14.3 mg. of galactose were found.

† 21.4 mg. of galactose were found.

picture is an increase of glucose concentration after absorption of galactose. This might be explained as due to (a) a conversion of galactose to glucose or (b) a stimulus of glycogenolysis by galactose.

Table IV gives the result of four control operations to test the effect of the anesthesia and the operation upon the level of blood glucose. In three of the four dogs there is a rise in blood glucose while in Dog 2 no effect can be noticed. Since amytal anesthesia and the operation without galactose injection produce a rise in blood glucose, with a variation in time of rise, in duration, and in

TABLE IV  
Effect of Amytal Anesthesia and Operative Shock on Blood Glucose

Dog No.	Weight kg.	Time after giving amytal	Time after end of operation	Blood glu- cos. mg. per cent	Increase over normal mg.	Remarks
1 ♀	12	0	0	63.0		Slow induction of anesthesia, very dilute amytal; much struggling; 46 mg. per kilo
		2 hrs. 15 min.	15 min.	89.5	26.5	
		3 " 30 "	1 hr. 30 "	93.5	30.5	
		5 " 35 "	3 hrs. 35 "	71.0	7.0	
2 ♂	16	0	0	63.0		Slow induction of anesthesia, very dilute amytal; no struggling; 56 mg. amytal per kilo
		2 hrs. 15 min.	30 min.	62.0	-1.0	
		3 " 30 "	1 hr. 45 "	59.5	-3.6	
		6 " 45 "	5 hrs.	62.0	-1.0	
3 ♂	15	0	0	54.5		Swift induction; no struggling; 60 mg. amytal per kilo
		1 hr. 18 min.	8 min.	59.5	5.0	
		2 hrs. 23 "	2 hrs. 13 "	66.0	11.5	
		3 " 18 "	3 " 8 "	90.5	36.0	
		5 " 18 "	5 " 8 "	55.5	1.0	
4 ♀	11.5	0	0	71.0		Pregnant animal; swift induction; no struggling; 60 mg. per kilo
		1 hr. 40 min.	30 min.	100.5	29.5	
		2 hrs. 40 "	1 hr. 30 "	62.0	-9.0	
		5 " 30 "	4 hrs. 20 "	75.2	4.2	

absolute amount, it is difficult to draw any definite conclusions about the effect of galactose on the glucose level of the blood.

#### SUMMARY

In dogs under amytal anesthesia, galactose appears in the blood and thoracic lymph when this sugar is absorbed from the intestine at a sufficiently rapid rate. The maximum concentration of galactose in the blood and lymph are directly related to the rate of absorption of this sugar. The galactose absorbed from the intestine by way of the thoracic duct is a very small per cent (less than 0.5 per cent) of the total galactose absorbed. The galactose absorbed through the thoracic duct in terms of per cent of the total sugar absorbed is greater with increased flow of lymph. During absorption of galactose the concentration of this sugar is greater in thoracic lymph than in femoral blood. The effect of galactose ingestion on the level of blood glucose varies widely in different animals.

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## PROTEINS OF YEAST (*SACCHAROMYCES CEREVISIÆ*)\*

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In the biological analysis of proteins yeast is commonly added to the diet in order to supply certain vitamins. Yeast protein is a good source of some of the nutritionally essential amino acids, and its addition to the experimental diet may in certain cases introduce errors by supplying amino acids in which the protein studied may be deficient. The magnitude of the error caused by the addition of yeast to the experimental diet could be estimated from the amino acid composition of the yeast proteins, but information on this point is very meager.

It is assumed that the protein in the yeast cell is present in conjugation with nucleic acid. At least, no one has obtained a preparation other than nucleoprotein or a derivative thereof. The proportion of nucleic acid to the protein varies widely in these preparations, judged from the amount of phosphorus found. In experimental diets whole yeast is generally added and not a yeast protein preparation. It has been found, however, that a direct acid hydrolysis of the whole yeast is not always suitable for amino acid determination. Cystine and histidine, for instance, were found for some reason not yet explained to be mostly decomposed.

As a rule, investigators in the past have used alkaline solvents in rather high concentration to extract the yeast protein, which undoubtedly changed the physical behavior and chemical composition of their preparations. Dreyer (1) used a 10 per cent ammonium carbonate solution and extracted 65.7 per cent of the

\* The material in this paper was presented before the Twenty-seventh and Twenty-eighth meetings of the American Society of Biological Chemists (*J. Biol. Chem.*, **100**, xxxiii (1933); **105**, xix (1934)).



total yeast nitrogen. He claimed that there are two proteins in the yeast, an albumin and a globulin, but he did not consider them as nucleoproteins, which was undoubtedly the case. Thomas (2) separated two preparations by partial autolysis, a paranucleo-protein, slightly soluble in 10 per cent sodium chloride solution and non-coagulable by heat, and an albumin coagulable in water at 40°. He analyzed these preparations for their nitrogen distribution according to Hausman's method and determined the basic amino acids by the method of Kossel and Kutscher. Meissenheimer (3) determined the amino acid content of an autolyzed yeast digest, from which he calculated approximately the amino acid composition of the yeast protein.

Both water and 10 per cent sodium chloride solution extract surprisingly small quantities of nitrogenous material from the untreated yeast cell; even a 0.2 per cent aqueous sodium hydroxide solution removes only about half the total nitrogen. By subjecting yeast cells to the action of ether prior to extraction with protein solvents Buchner and Gruber (4) in 1899 were enabled to remove a considerable part of the yeast protein. Later (1902), Schröder (5) by employing the same method prepared the water-soluble and heat-coagulable yeast protein, and determined its basic amino acid content. He also showed the presence of cystine, leucine, tyrosine, and phenylalanine. Recently, Chibnall and his associates (6) in their extensive work on the proteins of green leaves and grasses have developed and very successfully applied the use of ether in the plasmolysis of leaves for the separation of proteins.

In the work described in this paper ether treatment of the yeast previous to extraction by solvents was applied. The time of ether and yeast contact was varied in order to show the effect of the ether treatment on the distribution of nitrogen as extracted by different solvents.

The action of ether is 2-fold: first, it kills the yeast cell; and secondly, by removing lipid substances it makes the cell wall more permeable to the solvents used. The experiments were carried out at low temperatures (6–8°); however, autolytic and enzymatic activities were not prevented. The increase of the coagulable protein to a maximum after which less protein was obtained as the time of ether contact with the yeast cell was increased serves as an

indication that such reactions were taking place within the dead yeast cell. The protein preparations obtained are not to be considered as representing components of the living yeast cell, but are undoubtedly secondary products. This conclusion is reached from the nature of the results demonstrated in the experimental part.

Van Slyke's nitrogen distribution method is not applicable to the yeast nucleoprotein preparations because of the presence of purine and probably pyrimidine bases. Although the purines are unstable during acid hydrolysis at the acid concentration and time duration stipulated by Van Slyke, they are not decomposed completely. The presence of the bases interferes with the nitrogen distribution in practically every phase of the Van Slyke method. The dibasic amino acids were determined quantitatively, therefore, in the yeast protein preparations as well as in the whole yeast hydrolysate by the Vickery and Block (7) method as described in the experimental part. The cystine, tryptophane, histidine, and lysine content of the yeast protein places it in a favorable position among those considered of good quality. The amino acid composition of brewers' and bakers' yeast proteins is very similar.

#### EXPERIMENTAL

*Effect of Ether on Living Yeast Cell*—The bakers' and brewers' yeasts used in this investigation were prepared by two commercial concerns without the addition of fillers and washed free from nutrients. The time factor in the ether treatment of the yeast cell was established on both yeast preparations as follows: 10 to 12 gm. of the yeast press-cake were weighed into a 100 cc. centrifuge flask, and 30 to 40 cc. of ether were added to cover the yeast. The liquefaction of the yeast, hastened by occasional stirring, takes place within a few minutes. The ether was poured off after the required time interval, and four extractions, each lasting from  $\frac{3}{4}$  to 1 hour, were carried out on the residue with each solvent. The extracts were separated from the solids by centrifugation, syphoned off, and combined in a measuring cylinder, extracts of different solvents being kept separate. After the final extraction, the yeast residue was transferred to a Kjeldahl flask for the determination of unextracted nitrogen. All the samples and extracts

were kept at refrigeration temperatures of 6–8°. Nitrogen was determined in duplicate by the Kjeldahl method, aliquots from the extracts of each solvent being used (Table I).

As the time of the ether treatment increases, the unextracted nitrogen gradually diminishes, showing the source of extractable nitrogen. The water-soluble nitrogen increases with the time, while the salt-soluble nitrogen reaches a maximum and diminishes considerably toward the end of the experimental period. This behavior points to a continuous autolytic activity converting the original nucleoprotein of the yeast cell into the products here

TABLE I

*Extraction of Nitrogenous Substances by Different Solvents in Percentages of Total Nitrogen of Ether-Treated Yeast*

	Ether treatment of yeast	Water extract	10 per cent NaCl extract	0.2 per cent NaOH extract	Unextracted N	Heat-coagulable N in	
						Water extract	Salt extract
	hrs.	per cent	per cent	per cent	per cent	per cent	per cent
Bakers' yeast	Control	1.4	1.2	47.2	52.1		
	0.16	17.8	15.9	34.5	32.8	Trace	2.7
	1.0	19.5	21.0	27.5	34.0	"	3.5
	2.5	20.0	23.8	25.8	32.9		3.9
	18.0	30.1	25.8	33.4	13.4	9.9	8.3
	42.0	36.7	17.0	37.6	9.0	5.6	7.6
Brewers' yeast	Control	1.8	2.1	64.4	33.3		
	0.16	25.3	34.3	18.3	21.2		
	4.0	26.7	27.4	26.4	18.6		
	18.0	40.7	19.1	31.8	10.0		
	48.0	45.8	13.8	34.6	6.1		

separated. The higher the phosphorus content of the protein preparation, the more extensive was the proteolytic cleavage. The water-soluble protein preparation with the lowest phosphorus content should, therefore, represent in its amino acid composition the one nearest to that of the original yeast nucleoprotein.

*Yeast Protein Preparations*—The period of ether contact with the yeast which formed the largest amounts of coagulable protein was 18 to 20 hours. 500 gm. of yeast press-cake material were covered with ether, placed in a refrigerator for 18 hours, and occasionally stirred. After that the ether was syphoned off, and

1 liter of  $H_2O$  was added. The mixture was stirred vigorously and centrifuged. The water extraction was repeated twice. Each time approximately 1 liter of  $H_2O$  was added, and 1 hour was allowed for each extraction. The combined water extracts, amounting to approximately 2.5 liters, were acidified with 2 cc. of acetic acid and heated in a water bath. The water-soluble yeast protein coagulates at  $47-48^\circ$ , but to assure complete precipitation the temperature was brought to  $60^\circ$ . After cooling, the protein coagulum was separated by centrifugation. The precipitate was washed with distilled water and dehydrated in the usual way with alcohol followed by ether. The water-soluble yeast protein is also precipitable by ammonium sulfate at 65 per cent saturation.

The residue from the three water extractions was used for the preparation of the salt-soluble protein. The procedure was similar to that given above except that 10 per cent sodium chloride solution was used instead of water. The salt-soluble protein may be separated by heat coagulation ( $48-50^\circ$ ), by the addition of ammonium sulfate up to 60 per cent saturation, or by acidification (pH 4.0) with acetic acid. Neither water dilution nor dialysis is applicable for the separation of the salt-soluble protein; the isoelectric point, pH 4.7, as determined by the method of Csonka, Murphy, and Jones (8), is a further indication that we are not dealing with a true globulin.

The yeast residue from the water and salt extractions was finally extracted with 0.2 per cent aqueous sodium hydroxide, the same volume and time of extraction being used as in the case of the previous extractions. The combined alkali extracts were acidified to pH 4 by the addition of acetic acid. It is difficult to separate this precipitate by centrifugation because of the high viscosity of the extracts. The precipitate settles overnight, however, and the supernatant liquid can then be syphoned off and the precipitate washed in acidified water. At this stage, especially if alcohol is added, the protein precipitate may be easily separated by centrifugation. The yield of these protein preparations is far from being quantitative and should be considered as minimal (Tables II and III).

Several batches of yeast were worked up for protein preparations which were subjected to elementary analyses. Phosphorus was

determined volumetrically by the ammonium phosphomolybdate method after the protein material was ashed in the presence of magnesium nitrate and the residue dissolved in dilute nitric acid. Nitrogen was determined by the Kjeldahl and sulfur by the peroxide methods, Parr's heat ignition bomb being used (9).

TABLE II

*Yields of Protein Preparations Obtained from Ether-Treated (18 Hours) Brewers' Yeast and Calculated on 100 Gm. of Moisture-Free Yeast Containing 9.42 Gm. of Nitrogen*

Solvents used in successive extractions in order stated	Moisture- and ash-free protein	Protein N in per cent of total yeast N
	gm.	per cent
Water.....	8.24	13.4
10% NaCl.....	2.08	3.6
0.2% NaOH.....	5.38	9.5
Total.....	15.7	26.5

TABLE III

*Elementary Composition of Yeast Proteins Expressed in Percentages of Moisture- and Ash-Free Yeast Proteins*

	Bakers' yeast			Brewers' yeast		
	Nitro- gen	Phos- phorus	Sulfur	Nitro- gen	Phos- phorus	Sulfur
	per cent	per cent	per cent	per cent	per cent	per cent
Coagulum from water extract by heat.....	15.65	0.968	0.906	15.29	0.55	0.684
Coagulum from salt extract by heat.....	16.26					
Protein from salt extract by acidification.....	16.06	0.29	0.790	16.19	0.33	0.733
Protein from alkali extract by acidification.....	14.80	2.83	0.500	16.37	3.39	0.510

The method of Folin and Ciocalteu (10) was used for the tyrosine determination, that of Sullivan for cystine, and May and Rose's method with slight modification for tryptophane, as given in a previous communication (11) (Tables IV and V).

Vickery and Block's (7) method was adopted with certain

changes for the determination of the basic amino acids. The changes simplified manipulations, required shorter time, permitted the use of smaller quantities of silver nitrate reagent and protein material, and included a colorimetric method for the determination

TABLE IV  
*Amino Acids in Percentages of Moisture- and Ash-Free Yeast Proteins*

		Water-soluble protein by heat coagulation	Salt-soluble protein determined by		Alkali-soluble protein
			Heat	Acidification	
		per cent	per cent	per cent	per cent
Bakers' yeast	Cystine	0.44	0.82	0.87	0.69
	Tryptophane	2.66	3.18	2.96	1.79
	Tyrosine	4.79	3.96	4.44	3.11
Brewers' yeast	Cystine	0.49		0.61	0.31
	Tryptophane	2.67		2.49	1.68
	Tyrosine	4.11		3.85	3.93
	Arginine	3.50			2.82
	Histidine	1.38			Trace
	Lysine	4.50			4.53

TABLE V  
*Diamino Acids in Yeast Proteins*

	Schröder (water-soluble)	Thomas-Kolodziejska	
		Paranucleoprotein (salt-soluble)	Albumin (water-soluble)
	per cent	per cent	per cent
Arginine .....	3.22	3.58	3.95
	3.82		
Histidine .....	1.98	2.63	2.02
	2.36		
Lysine .....	8.68	4.09	7.14
	11.34		

of histidine based on the color test devised and published recently by Kapeller-Adler (12).

For the analysis approximately 5 gm. of protein material (more or less depending on the dibasic acid percentage of the material) are hydrolyzed for 36 to 40 hours at boiling temperature in 10

parts of 7 N sulfuric acid. The hydrolysate is then transferred quantitatively into a liter beaker, and most of the sulfuric acid is removed by the addition of slightly less than the calculated quantity of barium hydroxide dissolved in hot water, leaving the liquid slightly acid to Congo red indicator. The barium sulfate is removed by centrifugation and washed with 200 to 300 cc. of hot distilled water until the washings are practically colorless. The filtrate and washings are concentrated *in vacuo* and then transferred into a 500 cc. centrifuge bottle. The volume of liquid now should not be more than 100 cc. Concentrated silver nitrate solution is added until an excess of silver ion is present, as shown by the formation of a brown precipitate when saturated barium hydroxide solution is added to 1 drop of the clear supernatant liquid. Hot saturated barium hydroxide solution is added until pH 12 is reached, and the resulting mixture is cooled to room temperature and centrifuged. The precipitate contains the silver salts of histidine and arginine. The supernatant liquid, which should be slightly acidified with sulfuric acid, is set aside for lysine determine (Solution L). The silver precipitate in the centrifuge bottle containing both histidine and arginine is suspended in 80 to 100 cc. of distilled water, and concentrated sulfuric acid is added drop by drop until pH 3 to 4 is reached (blue to Congo red). It is stirred mechanically several times during a 1 hour period. A moistened strip of Congo red paper is placed in the upper part of the centrifuge flask to serve as a control in the next step of separating the histidine silver. Warm saturated barium hydroxide solution is added until the liquid changes the color of the test paper to purplish red. From here on cold saturated barium hydroxide is added until pH 7.4 is reached;<sup>1</sup> then the resultant mixture is centrifuged and the supernatant liquid set aside for arginine determination. In order completely to separate the arginine from the histidine the procedure described above is repeated twice in the same manner with the exception

<sup>1</sup> This is ascertained by placing a drop of the clear filtrate on a spot plate and adding brom-thymol blue indicator until a bluish green color is produced. Throwing down the silver precipitate by centrifugation greatly facilitates the settling of the subsequent precipitate formed by the addition of barium hydroxide solution; but the final test should be made after a thorough mixing.

that the precipitate is suspended each time in only about 50 cc. of water. The arginine-containing solutions are combined and designated Solution A.

*Determination of Histidine*—The histidine silver is decomposed with hydrogen sulfide after the precipitate has been suspended in distilled water and acidified with sulfuric acid. The silver sulfide is separated by centrifugation and the precipitate is washed twice, being suspended each time in 50 cc. portions of warm water saturated with hydrogen sulfide. The combined filtrate and washings containing the histidine are concentrated *in vacuo* to a small volume. This solution is now transferred into a 100 cc. Pyrex centrifuge tube, made up to 25 cc., and 1.25 gm. of concentrated sulfuric acid are added. The histidine is precipitated by adding an aqueous solution containing 10 per cent mercuric sulfate and 5 per cent by weight of sulfuric acid (Hopkins' reagent). After it has stood overnight in the refrigerator, it is centrifuged and the supernatant liquid discarded. The mercuric salt is decomposed with hydrogen sulfide in a manner similar to that described above for the silver histidine. The combined filtrates and washings are concentrated *in vacuo* to a volume of 25 to 30 cc., transferred into a 100 cc. Pyrex centrifuge tube, and brought to neutrality with saturated barium hydroxide solution. Copper carbonate is added in excess, and the tube is placed for  $\frac{1}{2}$  hour in a boiling water bath, after which it is kept in the refrigerator overnight. It is then centrifuged, the supernatant liquid is poured off, and the precipitate is washed twice by suspending it in 5 cc. of cold water. The copper is removed with hydrogen sulfide from the combined liquids in the manner given for the removal of mercury or silver. The liquid is now concentrated *in vacuo* and made up to a volume of 75 or 100 cc. (Solution H).

The Kapeller-Adler (12) colorimetric test is employed for the histidine determination. 1 to 2 cc. of the purified histidine Solution H are measured into a Pyrex test-tube, and 1 cc. of a standard histidine solution<sup>1</sup> into another tube. The bromine reagent is added drop by drop to both test-tubes which are shaken vigorously until the liquids show a light yellowish tint that should remain permanent for 10 minutes. Then add 2 cc. of the ammoniacal ammonium carbonate mixture to each and place the tubes in a boiling water bath for 5 minutes. Cool, and transfer both stand-



ard and unknown to 10 cc. measuring cylinders. Bring the standard up to 4 cc. volume with the ammoniacal ammonium carbonate reagent and the unknown to a volume between 4 to 8 cc., which gives a reading within  $\pm 2$  mm. of that of the standard set at 20 mm. One preliminary determination is sufficient to make adjustment to fulfil these requirements.

Objection may be raised to the application of this colorimetric method to the analysis of protein preparations which may contain purines. However, when this method was applied in the analysis of the whole yeast cell, as referred to later, a negative result for histidine was obtained, showing that purines were not a color-contributing factor as the method was applied in this investigation.

*Determination of Arginine*—Solution A, which was immediately acidified with dilute sulfuric acid, is concentrated *in vacuo*, transferred, and, if necessary, filtered into a 500 cc. centrifuge bottle. The volume, including washings, required to transfer the concentrate should be not more than 50 cc. A test should also be made for the presence of excess silver ion and, if necessary, sufficient silver nitrate is added. The arginine silver is then precipitated by adding an equal volume of hot saturated barium hydroxide (and more, if necessary, to reach pH 12), centrifuged, and the supernatant liquid added to Solution L. The precipitate is suspended in 30 to 40 cc. of distilled water, the mixture acidified with sulfuric acid to pH 4, and the arginine silver is reprecipitated at pH 12 as given above. The arginine silver precipitate is now suspended in 50 cc. of cold saturated barium hydroxide solution and centrifuged. This one washing is generally enough to free it from nitric acid. The arginine silver precipitate is now decomposed with hydrogen sulfide according to the procedure described for the histidine. In an aliquot the nitrogen content is determined by the Kjeldahl method, and in the remaining portion most of the sulfuric acid is precipitated with barium hydroxide, leaving the solution faintly acid. The solution is then concentrated to a small volume and the required amount of flavianic acid (nitrogen  $\times 5.61$ ) is added. When the solution is brought to boiling, the arginine monoflavianate is obtained as an orange precipitate. The precipitate, after standing overnight in a refrigerator, is filtered off by suction and washed first with a few cc. of cold water, then with alcohol, then with ether, and finally dried

at 105°. The weight of the arginine monoflavinate multiplied by 0.3566 gives the amount of arginine in the aliquot used.

*Lysine Determination*—Solution L combined with the washings is acidified with sulfuric acid and the silver removed by hydrogen sulfide. The clear liquid, including washings, is freed from mercuric sulfate and barium and concentrated *in vacuo* to a volume of approximately 50 cc. Concentrated sodium hydroxide solution is added to a slightly alkaline reaction and then an equal volume of alcohol. The ammonia thus liberated is removed by distillation *in vacuo*. The concentrate is now transferred to a 500 cc. Pyrex centrifuge tube and neutralized with sulfuric acid, and then enough concentrated sulfuric acid is added to make the final concentration 5 per cent by weight. The lysine is precipitated by the addition of an excess of phosphotungstic acid (100 per cent aqueous solution), and the precipitate is redissolved by heating it in a water bath. After standing in a refrigerator overnight, the lysine phosphotungstate precipitate is removed by centrifugation and washed twice with 25 to 30 cc. of chilled 2.5 per cent phosphotungstic acid in 5 per cent sulfuric acid. It is then dissolved in 50 per cent aqueous acetone solution and decomposed by an excess of warm saturated barium hydroxide solution at pH 10. The barium phosphotungstate precipitate is washed twice with cold saturated barium hydroxide solution, and the combined liquids are freed from barium by the addition of sulfuric acid in slight excess. The nitrogen content is determined in an aliquot by the Kjeldahl method, and the remainder of the solution is concentrated in the presence of barium carbonate (13) to a small volume. The precipitate is filtered off and washed. The combined liquids are concentrated to a sirupy consistency, preferably *in vacuo*, with a 100 cc. Claisen flask with a removable ground jointed bulb. Absolute alcohol is added to the sirupy residue until a slight turbidity is formed, which disappears after the addition of a few drops of water. At first only two-thirds of the required amount of picric acid (calculated by multiplying the nitrogen content of the aliquot used by 8.1) dissolved in hot absolute alcohol is added (13). The lysine picrate thus formed is allowed to stand in a refrigerator overnight and then centrifuged, the mother liquor being used to transfer the precipitate adhering to the Claisen flask quantitatively into the small centrifuge tube. The crude lysine

picrate is suspended in a mixture of 5 cc. of absolute alcohol and ether, and the washing is repeated twice. The remaining one-third of the calculated amount of picric acid is added to the mother liquor, and the precipitate, if any, treated as described above. Finally, both precipitates of the crude lysine picrates are combined and recrystallized from a small amount of water. After standing overnight at room temperature, the precipitate is filtered off, dried, and weighed. The solubility correction of 0.54 gm. per 100 cc. is added to the lysine picrate actually separated.

*Amino Acid Content of Whole Yeast Cell*—Cystine is a constituent of glutathione, which was shown by Hopkins (14) to be present in the yeast cell. For this reason it was of especial interest to ascertain the quantity of cystine in the whole yeast cell, since the amount present as part of the yeast protein molecule does not represent all the cystine. It was found, however, that the cystine is decomposed by acid hydrolysis of the yeast. Aliquots were taken, therefore, from the extracts obtained in the studies of ether effect on the brewers' yeast (Table I). The aliquots from the 18 hour experiment were slightly acidified with sulfuric acid and evaporated to a volume of 20 cc. in a flask in which later the hydrolysis was accomplished, after sufficient sulfuric acid had been added to bring the acid concentration to 6 N. A value of 0.3 per cent for cystine was found as determined by Sullivan's method as modified by the author (11). Bakers' yeast treated similarly was found to contain 0.27 per cent cystine, calculated on moisture-free yeast.<sup>2</sup> Arginine and lysine were determined by the method described above in a 50 gm. sample of brewers' yeast press-cake after hydrolysis for 42 hours at boiling temperature with 100 cc. of 7 N sulfuric acid. 7 gm. of concentrated sulfuric acid had been added previously to take care of the moisture in the press-cake. The analysis, calculated on a moisture-free basis, showed that bakers' and brewers' yeasts contained 1.32 and 1.37 per cent of arginine and 2.15 and 2.61 per cent of lysine, respectively. Histidine was not found in the hydrolysate. This negative result may explain Schenk's (16) erroneous statement that the yeast cell lacks histidine. An attempt to estimate the quantities of tryptophane and tyrosine in the whole yeast, according to the methods

<sup>2</sup> Prunty (15) found in two samples of dried brewers' yeast, which he analyzed, 0.92 and 0.52 per cent cystine.

of May and Rose, and of Folin and Ciocalteu, respectively, was unsuccessful.

#### SUMMARY

Cystine, tryptophane, tyrosine, arginine, histidine, and lysine were determined quantitatively in protein preparations obtained from bakers' yeast and from brewers' yeast. It was found that cystine and histidine undergo a change when yeast is hydrolyzed by acid. Because of the high percentages of nutritionally essential amino acids in yeast proteins the supplementing effect of yeast when added to experimental diets should be taken into consideration.

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## THE DETERMINATION OF INORGANIC SULFATE IN THE SERUM OF NORMAL PERSONS

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In recent years a number of methods have been published for the determination of inorganic sulfate in small quantities of blood serum. Each of these methods utilizes the same preliminary technique of precipitating the sulfate as benzidine sulfate by the addition of a solution of benzidine in acetone to a trichloroacetic acid filtrate of serum. It is only in the manipulation of the precipitated benzidine sulfate for its final estimation that the several methods differ. Colorimetric procedures are employed by Yoshimatsu (1), Wakefield (2), Hubbard (3), and Cuthbertson and Tompsett (4). Cope (5) determines the sulfate by microtitration with sodium hydroxide with the aid of a Rehberg microburette. Power and Wakefield (6) oxidize the benzidine sulfate with an excess of potassium dichromate and determine the excess of dichromate. The values obtained for inorganic sulfate concentrations in normal serum in these various procedures differ widely from each other and from the values obtained by the earlier nephelometric method of Denis (7) (see Table I).

After studying the factors influencing the precipitation of sulfate as benzidine sulfate, the present authors have been led to the conclusion that the fundamental error of these methods is the assumption that pure benzidine sulfate can be quantitatively precipitated from serum filtrates in the manner described. Sulfate is precipitable as benzidine sulfate in the presence of a large excess of benzidine ions. Its solubility is further diminished by the presence of acetone. A sufficiently high concentration of benzidine ions is possible only in the presence of a strong acid such as hydrochloric or trichloroacetic. If the acidity is too great,

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the sulfate precipitation is incomplete and may, in fact, in solutions of such minute concentrations as are found in blood filtrates, be prevented altogether. If, on the other hand, the acidity is not great enough, other ions are also precipitated, particularly phosphate and chloride. If the acidity is that of the range of acetate buffers (pH of 4.5 or higher), free benzidine is also precipitated. The quantity of precipitated free benzidine may be so great as to diminish effectively the excess of benzidine ions required to produce complete precipitation of the sulfate.

When benzidine in acetone is added to a sulfate solution containing trichloroacetic acid, benzidine ions are formed which pre-

TABLE I  
*Concentration of Normal Serum Inorganic Sulfate As Determined by Various Investigators*

Author	Precipitating agent	Method	Range of values
			<i>mg. S per 100 cc. serum</i>
Denis (7) . . . . .	Barium	Nephelometric	0.5-1.0
Kahn and Postmontier (8) . . . . .	"	Volumetric	1.0-3.0
Yoshimatsu (1) . . . . .	Benzidine	Colorimetric	2.0-3.3
Loeb and Benedict (9) . . . . .	Barium	Gravimetric	0.7-1.6
Wakefield (2) . . . . .	Benzidine	Colorimetric	0.2-0.9
Cuthbertson and Tompsett (4) . . . . .	"	"	0.1-0.5
Wakefield, Power, and Keith (10) . . . . .	"	Oxidimetric	0.8-1.7
Macy (11) . . . . .	"	"	1.1-1.9
Hoffman and Cardon . . . . .	"	"	0.3-1.1

cipitate the sulfate present; but in the presence of phosphate and chloride, some benzidine phosphate and chloride are included in the precipitate. When such a precipitation is effected in a trichloroacetic acid filtrate of serum, probably traces of many other substances are also present in the precipitate; for the precipitate formed is a discolored amorphous mass in no way resembling the discrete, satiny, white crystals of benzidine sulfate readily obtainable by adding benzidine chloride in hydrochloric acid to a sulfate solution. This amorphous precipitate gives qualitative tests for both phosphate and chloride. The higher the concentration of phosphate in the serum, the greater is the contamination of the

precipitate with benzidine phosphate. The extent of the error involved will vary with the type of estimation, depending upon whether the benzidine portion is being analyzed as in colorimetric or oxidimetric methods, or whether the acid portions are being determined, as in the usual volumetric procedure of titrating the precipitate in boiling water with sodium hydroxide. Using the latter method and working with large quantities of both synthetic inorganic solutions and trichloroacetic acid filtrates from pooled serum, the authors were able to estimate the degree of error due to the contamination of benzidine sulfate with other substances. This error was found to be of the order of 35 per cent for inorganic solutions with small concentrations of phosphate and for normal serum, while for serum of patients with uremia, the error was sometimes as high as 300 per cent.

To avoid these difficulties, the authors have developed a protein-free, phosphate-free filtrate of serum, from which sulfate can be quantitatively precipitated with the usual benzidine chloride reagent and acetone. Such a filtrate is obtained by a modification of the senior author's method (12) of preparing a filtrate for serum total base determination. The precipitate of benzidine sulfate thus obtained is white, discrete, crystalline. In fact, its purity has made it difficult to wash it with acetone without loss, a difficulty which has been overcome only by utilization of a specially designed centrifuge tube and a special technique for washing.

For the estimation of the quantity of benzidine sulfate, it has been found convenient to adopt the permanganate oxidation method used by Kramer and Tisdall (13) for the determination of potassium. The amount of potassium permanganate required for complete oxidation of benzidine is determined empirically by running complete analyses on solutions of sodium sulfate, the concentrations of sulfate in which are of the order found in the blood filtrates (see Fig. 1).

### *Method*

#### *Reagents*

1. Ferric chloride. 80 cc. of a stock solution of 10 per cent ferric chloride in 0.2 N HCl are diluted to a liter with distilled water.



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2. Ammonium hydroxide-ammonium acetate solution. 2 gm. of ammonium acetate and 14.7 cc. of  $N$   $NH_4OH$  are made up to a liter with distilled water.

3. Benzidine chloride solution. 4 gm. of benzidine of highest purity are added to 45 cc. of  $N$   $HCl$  in a 250 cc. volumetric flask, diluted to about 150 cc. with distilled water, and agitated until dissolved. The solution is made up to the mark and filtered. A small portion of the solution is refiltered each time before using.

4. Potassium permanganate 0.1  $N$ .

5. Potassium permanganate 0.02  $N$ .

6. Sodium oxalate 0.1  $N$ .

7. Sulfuric acid 4  $N$ .

8. Redistilled acetone. Acetone U.S.P. may be used if redistilled with a fractionating column.

9. Acetone 90 per cent by volume.

### *Procedure*

4 cc. of serum are measured into a Pyrex test-tube which has been marked for 18 cc., and treated with stirring with 2 cc. of ferric chloride reagent and 2 cc. of ammonium hydroxide-ammonium acetate reagent. The tube is placed in a boiling water bath for 2 minutes. At this point, a yellow coagulum is formed, containing all the serum protein and fat, all the phosphate as ferric phosphate, and all the excess iron as basic ferric acetate. A clear interstitial fluid containing only a trace of pigment can be seen through the coagulum. If, as sometimes happens when serum from patients with marked acidosis or with low serum protein is used, the proportion of reagents is not exactly correct, the interstitial fluid will not be clear. Under these circumstances, 1 drop of 5 per cent ammonium acetate solution should be added to the boiling mixture, at which addition the coagulation will invariably become complete.

The test-tube is cooled to room temperature, and 90 per cent acetone is added to the 18 cc. mark. The material is then thoroughly mixed with a stirring rod and filtered through an ashless filter paper into another test-tube. The first 2 or 3 cc. of the filtrate should be poured back for refiltration, but the filtration of 10 cc. should be made in a minimum time so as to make con-

stant the error due to evaporation of the water-acetone mixture. The filtrate should be water-clear.

10 cc. of the filtrate are pipetted into a specially designed 15 cc. conical centrifuge tube. This tube has been pulled out at the conical end to a parallel-sided capillary tube, 5 mm. long and not more than 2.5 mm. in diameter. Only such a tube was found practicable for washing the small quantity of discrete crystals of benzidine sulfate without loss. To the filtrate are added 1 cc. of 90 per cent acetone and then, drop by drop, 1 cc. of the benzidine chloride reagent. The mixture is then stirred with a fine glass rod tipped with platinum wire. The stirring rod is washed off with 90 per cent acetone. The tube and its contents are placed in a refrigerator for an hour. The material is then centrifuged for 15 minutes at about 2000 R.P.M. The supernatant fluid is removed by inverting the tube and allowing it to drain for about 2 minutes. So long as the capillary end of the tube remains filled with fluid, the precipitated benzidine sulfate will not be lost. The precipitate is washed with about 1 cc. of 90 per cent acetone with the aid of the platinum-tipped stirring rod, and the tube is again centrifuged. Two more washings are made, this time with 100 per cent acetone, about 4 cc. being used each time. The centrifugations of the washings need not be continued for more than 5 minutes.

After the third washing, the precipitate is stirred up with about 2 cc. of water and the tube is then placed in a boiling water bath for at least 20 minutes to remove the last traces of acetone. At no time in the manipulation of the precipitate should it be allowed to become dry; for drying promotes clumping of the crystals, which makes them difficult later to dissolve and be oxidized. To the watery suspension are added exactly 1 cc. of 0.1 N permanganate and 1 cc. of 4 N sulfuric acid. The mixture is kept in the boiling water bath for 2 minutes with constant stirring with the platinum-tipped stirring rod. The excess permanganate is now reduced by adding exactly 1 cc. of 0.1 N sodium oxalate with constant stirring until all particles of  $\text{MnO}_2$  are dissolved, leaving a colorless solution. The excess oxalate is now titrated hot with 0.02 N permanganate in a microburette until the first appearance of pink.

A blank determination should be made on the reagents used.

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Since the sodium oxalate remains quite stable, whereas the potassium permanganate solutions, even though prepared with the usual precautions, alter slightly with time, the relative constancy of the value of the blank can be used as a check on the 0.1 N permanganate. Only the 0.02 N permanganate need be frequently standardized against the oxalate.

The amount of permanganate required to oxidize the benzidine equivalent of 0.01 mg. of S was found to be 0.68 cc. of 0.02 N

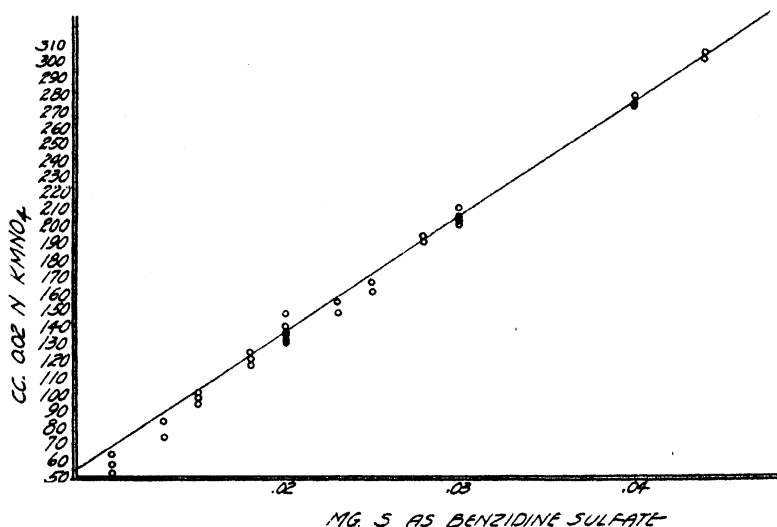


FIG. 1. The amounts of  $\text{KMnO}_4$  required for the oxidation of varying amounts of benzidine sulfate, as determined empirically. The straight line, 0.68 cc. of 0.02 N  $\text{KMnO}_4$  = 0.01 mg. of S, represents the average values obtained for quantities of benzidine sulfate between 0.02 and 0.04 mg. of S per 100 cc.

$\text{KMnO}_4$  (see Fig. 1). This value should be determined empirically by carrying out a series of analyses in the above manner on inorganic sulfate solutions, containing from 0.01 to 0.04 mg. of S in 10 cc. of 60 per cent acetone. In this way, the personal errors of the determination are minimized. Fig. 1 shows that sulfate determinations by this method are likely to be inaccurate if the amount of sulfate determined in the 10 cc. of filtrate is much less than 0.02 mg. of S.

There is a constant positive error in the determination which makes the values obtained 3 per cent too high on an average. This error is caused by three factors: the shrinkage of volume when the serum with its precipitate is mixed with acetone, the volume occupied by the precipitated protein, and the evaporation of water-acetone solution during filtration. The value of this error was determined by analyzing the filtrates for total base and comparing the values with those obtained by the senior author's method for serum total base.

The calculation of the serum inorganic sulfate concentration is best illustrated by the following example. The final titration of 0.02 N  $\text{KMnO}_4$  was 1.26. The blank was 0.06 cc. 4 cc. of serum were used, and a filtrate representing 10/18 of this was analyzed for sulfate. The permanganate equivalent for 0.01 mg. of S was 0.68 cc. The error of the determination was 3 per cent.

$$0.01 \times \frac{1.26 - 0.06}{0.68} \times \frac{18}{40} \times \frac{97}{100} \times 100 = 0.77 \text{ mg. S per 100 cc.}$$

or

$$(1.26 - 0.06) \times 0.642 = 0.77 \text{ mg. S per 100 cc.}$$

*Comment on the Method*—The quantity of serum and the extent of the dilution to form the protein-free, phosphate-free filtrate have been adjusted to give a filtrate containing about 0.02 mg. of S per 10 cc. Such an adjustment permits accurate determinations with a quantity of filtrate that can conveniently be handled in a small centrifuge tube. For normal serum, 4 cc. of serum must be used. However, in analyzing the serum of uremia patients, which contains from 10 to 16 times as much inorganic sulfate as normal serum, the quantity of serum or filtrate must be chosen to provide not more than 0.04 mg. of S for analysis. More than this amount will give too bulky a precipitate of benzdine sulfate for convenient handling. The authors found it convenient in these cases to use 4 cc. of serum as for the normal, but to take only 2 or 3 cc. of filtrate for analysis, diluting to 10 cc. with 60 per cent acetone.

The addition of acetone directly to the serum containing the coagulated protein combines in one stroke the proper dilution of the serum and the addition of acetone for the precipitation of

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sulfate. It also serves to remove any trace of unprecipitated protein from solution. It has one distinct disadvantage; that is, it redissolves some lipid material which may be precipitated along with the benzidine sulfate where the benzidine chloride reagent is added. Even though the lipid substances are later redissolved during the washings with acetone, their presence causes trouble by making the crystals of benzidine sulfate adhere to the stirring rod. It is to avoid this precipitation of lipid that 1 cc. of 90 per cent acetone is added to the filtrate before the addition of benzidine chloride.

The first washing is done with 90 per cent acetone instead of pure acetone to avoid the precipitation of benzidine chloride,

TABLE II

*Comparison of Values Obtained for Serum Inorganic Sulfate by Authors' Method with Those by Macro volumetric Analysis of Large Quantities of Pooled Serum from Uremia Patients*

Sample No.	Volumetric method	Authors' method
	<i>mg. S per 100 cc. serum</i>	<i>mg. S per 100 cc. serum</i>
1	9.45	9.10 9.25
2	6.78	6.62 6.50
3	7.60	7.62 7.30

which is insoluble in 100 per cent acetone. After the first washing, there is so little benzidine chloride present in the centrifuge tube that further washing can be carried out safely with 100 per cent acetone.

The accuracy of the method as applied to serum was tested in two ways. First, benzidine sulfate, precipitated by the above method in large quantities of filtrate from pooled serum of uremic patients, was analyzed by the usual volumetric method by titration with 0.02 N NaOH in boiling water, and the results obtained were compared with those obtained by permanganate oxidation. Secondly, known quantities of sulfate were added to serum and their quantitative recovery determined by the above method. Tables II and III show the results of these experiments.

*Inorganic Sulfate Concentration in Normal Serum*

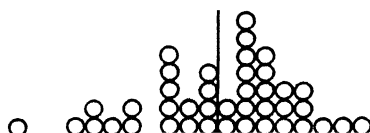
The concentration of serum inorganic sulfate was determined in forty-two normal men and women ranging in age from 21 to 47 years. The average concentration was 0.77 mg. per 100 cc. of

TABLE III

*Recovery of Inorganic Sulfate Added to Serum*

The results are expressed in mg. of S per 100 cc. of serum.

Sample No.	Inorganic sulfate	Added sulfate	Total sulfate	
			Calculated	Found
1	0.60	1.84	2.44	2.24
2	0.60	3.68	4.28	4.11
3	0.78	1.84	2.62	2.82
4	0.90	3.68	4.58	4.31
5	1.05	1.30	2.35	2.45



.38 .46 .54 .62 .70 .78 .86 .94 1.02 1.10 1.18

FIG. 2. Frequency chart of individual serum inorganic sulfate concentrations in forty-two normal persons. The mean = 0.77 mg. of S per 100 cc. of serum; standard deviation  $\pm 0.16$  mg. per 100 cc.

serum, with a standard deviation of  $\pm 0.16$  mg. The minimum value was 0.34 and the maximum 1.09 mg. of S per 100 cc. The distribution of the values when charted in a frequency chart (Fig. 2) shows a fair approximation to a probability curve. The

values have a broad distribution similar to that found for urea (14), which is what might be expected, since, like urea, sulfate is a metabolic waste product. The average value for serum inorganic sulfate concentration by this method is somewhat lower than that given by Power and Wakefield and other recent investigators (see Table I), but the differences are very much greater in serum of patients with Bright's disease, a study of which is being published separately. It will be noted that, while the values obtained by the present method are lower than those obtained by using a trichloroacetic acid filtrate, they agree essentially with the values originally obtained by Denis, who used a difficult nephelometric determination of a suspension of barium sulfate.

#### SUMMARY

Previous methods for the determination of serum inorganic sulfate which depend upon the precipitation of benzidine sulfate by the addition of benzidine in acetone to a trichloroacetic acid filtrate of serum give erroneous results. The values obtained are from 35 to 300 per cent too high, because of the inclusion of other substances with the benzidine sulfate precipitate.

A new method for serum inorganic sulfate is described which avoids these errors by using a specially prepared phosphate-free, protein-free filtrate from serum, in which sulfate can be precipitated by using the ordinary benzidine chloride solution in hydrochloric acid.

The serum inorganic sulfate concentration has been determined by this method in forty-two normal persons. The average value was 0.77 mg. of S per 100 cc., with a standard deviation of  $\pm 0.16$  mg.

These values are lower than those obtained by recent investigators, but agree essentially with those originally obtained by Denis.

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# **SOME OBSERVATIONS ON THE PHYSIOLOGICAL ADJUSTMENT OF THE ALBINO RAT TO A DIET POOR IN SALTS WHEN EDESTIN IS THE SOURCE OF DIETARY PROTEIN \***

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(Received for publication, March 4, 1935)

In a previous investigation, certain phases of the adjustment of the albino rat to a complete withdrawal of the inorganic constituents of the diet were reported (Swanson and Smith, 1932). Comparative studies of blood pictures of rats grown on salt-free and adequate rations, respectively, have shown that the defective diet induced marked changes in the blood stream when casein served as the source of dietary protein. When fed this ration, the animals developed definite polycythemia. During the 3 months of observation, the normal increment in the number of erythrocytes was 2 million, whereas an increase of 4.5 million occurred in the rats whose food salts had been strictly limited. The red cells became very small in size and the concentration of hemoglobin was reduced. It was also proved that alterations in blood volume were not responsible for the increased erythrocytic concentration.

Inasmuch as casein is a conjugated protein containing phosphorus as an integral part of its molecule, it was realized that an abnormally high ratio of phosphorus to other ash constituents characterized the small inorganic residue of the basal ration. In an effort to formulate a diet containing a minimal amount of salts without any one element present in preponderance, the casein of the ration was replaced by the simple protein, the globulin edestin. By this substitution, the total ash content of the diet

\* A preliminary report was presented before the meeting of the American Physiological Society at Philadelphia, April, 1932.

was reduced from 0.50 to 0.27 per cent. Feeding trials with a limited number of animals indicated that the rat did not react to the low salt edestin diet as it had to the ration containing the protein, casein. The present investigation was, therefore, planned to study in detail the specific physiological adaptations induced in the rat by the administration of two low salt diets containing casein and edestin, respectively, as the sole source of protein.

#### EXPERIMENTAL

The experiment was divided into two parts. The first dealt with the effect of feeding the two low ash diets on blood constituents of mature rats, the age and size of those described by Swanson and Smith (1932, *a*), as contrasted with the blood pictures characteristic of the control groups of rats grown on the same diets, each fortified by an adequate salt mixture.

The erythrocytes were enumerated and the red cell volume, the concentration of hemoglobin, and the per cent of reticulocytes determined at the end of the experimental period.

The second part of the investigation dealt with measurements of the average intake of water and the average output of urine. In the experiments relating to the condition of the blood, it was noted that the low salt edestin diet seemed to exert a specific diuretic effect. A carefully controlled experiment was, therefore, planned to determine whether the water metabolism of the rat was definitely influenced by the consumption of food containing minimal amounts of the essential dietary salts.

*Blood Studies*.—Forty male rats of Wistar strain, inbred by brother and sister matings for 55 generations, were used for observation. They were 37 days old when the feeding of the experimental diets was inaugurated, had an average weight of 104 gm., and were maintained upon the various test régimes for a period of 90 days. The rats were distributed as uniformly as possible into four groups. Group I received a diet poor in salts designated as Diet 5, in which edestin was the protein; Group II, the same diet but with casein substituted for the edestin (Diet 3); Group III, a control group, was given Diet 5 fortified by a salt mixture, supposedly quantitatively and qualitatively adequate (Diet 6); and Group IV, the second control series, was fed a ration, designated as Diet 2, that consisted of Diet 3 supplemented by the same

salt mixture given to Group III. The composition of each diet fed to the animals during the test period is shown in Table I.

Yeast that was used in the previous experiment when a polycythemic condition in mature rats was first reported (Swanson and Smith, 1932, *a*) was still available for this study. Casein, with an ash residue of 1.9 per cent (Shaw, 1920), and extracts of vitamins B and E were prepared in the manner described in the original study. The edestin incorporated into the two diets formulated with this protein was of the highest purity Pfanstiehl grade.<sup>1</sup> Upon ashing by the calcium acetate method (Shaw, 1920), the

TABLE I  
*Composition of Experimental Diets*

Dietary components	Experimental animals		Controls	
	Group I, fed Diet 5	Group II, fed Diet 3	Group III, fed Diet 6	Group IV, fed Diet 2
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Low ash casein.....		18		18
“ “ edestin.....	18		18	
Dextrin.....	55	55	51	51
Hydrogenated fat (Crisco)....	27	27	27	27
Salt mixture*.....			4	4

200 mg. dried yeast

3 drops wheat germ oil

1 cc. alcoholic extract of wheat germ

5 drops cod liver oil

Daily adjuvants

for all groups

equivalent to

30 mg. ash

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 557 (1919).

low salt edestin diet had a residue of 0.27 per cent; the low salt casein ration, 0.50 per cent.

The precautions necessary in the selection and care of the animals in this type of experiment were observed (Swanson and Smith, 1934). The methods employed for securing the sample of blood for the enumeration of the erythrocytes, and for the determination of the volume occupied by the red cells have also been described elsewhere (Swanson and Smith, 1932, *a*).

<sup>1</sup> Purchased from the Arthur H. Thomas Company, Philadelphia.

The Bürker method<sup>2</sup> was used for the estimation of hemoglobin. The standard employed in the colorimetric comparison was a reduced hemoglobin solution prepared by Dr. Bürker and hermetically sealed in the standard cup of the Leitz-Bürker colorimeter. The definite concentration of hemoglobin in the standard had been determined by chemical and spectrographic tests. The color of the solution was stable; no change or fading occurred over a period of a year. Determinations by this method very closely checked those made by the acid hematin method (Cohen and Smith, 1919).

The percentage of reticulocytes was ascertained by the examination of "permanent" blood smears prepared by the Cunningham (1920) technique. The stains were very carefully made, and attention was given to such details as the use of chemically clean apparatus, uniform coating of slides with brilliant cresyl blue, the use of unclotted samples, and the use of a droplet of blood of the proper size. Two smears were made from the blood of each rat; 1000 cells were counted in each, with oil immersion magnification. In the final enumeration, the field-constricting disk described by Orten and Smith (1934, *a*) was used, thereby restricting the field to 5 to 10 cells. The blood of either five or six rats from each experimental group was studied for the proportion of reticulocytes. The animals chosen represented four litters, distributed equally over the four variations of the experiment.

*Measurement of Water Intake and Urinary Output*—The number of animals in each group of this experiment varied from six to seventeen. The rats were housed individually in cages with wide meshed screen bottoms. The cage was suspended over a large glass funnel whose tip touched the bottom of a long stemmed Gooch funnel inserted by means of a stopper in a graduated cylinder containing a layer of mineral oil. The number of cc. of urine excreted were read directly at the same time every day, after which the apparatus was thoroughly cleaned. A perforated plate in the large funnel prevented fecal contamination of the urine. The cage was equipped with a glass fountain into which 100 cc. of water were measured daily. At the end of each period, the quantity of water remaining was determined.

<sup>2</sup> See directions for Leitz colorimeter, booklet E. No. 2363 (1928), Ernst Leitz Optical Works.

### Results

*Growth*—The two groups of rats fed, respectively, the two deficient rations grew at a retarded rate for the first 30 days of the experimental period; thereafter, the average body weight of the two groups was held at 147 gm. ( $\pm 14$  gm.)<sup>3</sup> for the remaining 60 days of the experiment. The average gain in body weight during the experimental period of 12 weeks was 43 gm. The correlation between inhibited growth and the severity of the polycythemia induced by the elimination of the ash constituents of the diet has been reported (Swanson and Smith, 1934). It is of importance to emphasize the fact here that the average growth curves of the two groups of rats fed the salt-poor diets were strikingly similar and that, therefore, differences observed in the blood pictures could not be ascribed to variance in the growth response. On the other hand, the average gain in weight of the rats fed the two adequate diets during the experimental period of 3 months was 114 gm. The rats fed the synthetic casein diet grew slightly better than did the rats given the adequate edestin food.

*Blood Pictures*—In the present experiment there was used a different strain of animals than those employed in the earlier studies (Swanson and Smith, 1932, *a*). It is, therefore, of interest to note from Table II that the low ash casein diet again definitely stimulated red cell formation to approximately the same degree that it did in the original investigation. However, it is remarkable that the mere substitution of casein by edestin so definitely reduced the erythropoietic qualities of the ration that the rat maintained a normal red cell concentration; *i.e.*, 9.5 million cells per c.mm. Almost an identical number of erythrocytes were present in the respective bloods of the rats fed the two control diets. The hematogenic effect of Diet 3 was, therefore, apparently induced by some sort of dietary unbalance brought about by the use of a salt-free casein unfortified by an adequate mineral mixture.

From considerations other than the number of erythrocytes present, the blood of the rats given the low salt edestin ration was less affected by the defective dietary regimen than was that of the casein-fed animals. For instance, the salt-poor edestin diet did not produce as pronounced a regression in size of cells. Calcula-

<sup>3</sup> Standard deviation.

tions for corpuscular volume (Wintrobe, 1929) show that the average size of the erythrocyte in these animals was 48 cubic microns, and those of the normal rat, 55 cubic microns; whereas the cells of the rats fed the low salt casein diet measured only 39 cubic microns (Table II). The quantity of hemoglobin in the blood of the rats given the inadequate edestin diet was somewhat less than normal but the individual cells contained more hemoglobin than did the erythrocytes in the blood of the rats composing

TABLE II  
*Blood Pictures of Rats Fed Various Experimental Diets*

Ration	No. of rats	Erythrocytes per c.mm.	Reticu- locytes	Erythro- cyte volume	Corpus- cular volume	Hb per 100 cc.	Corpus- cular Hb	Satura- tion with Hb
		million	per cent	per cent	cu. microns	gm.	gm. $\times$ 10 <sup>-12</sup>	per cent
Low ash edestin (Group I).	11	9.5 $\pm$ 0.68*	3.1	46 $\pm$ 2.1	48	13.1 $\pm$ 1.5	13.5	28.1
Low ash casein (Group II).....	11	11.5 $\pm$ 0.83	3.7	45 $\pm$ 4.7	39	12.9 $\pm$ 1.7	11.2	28.6
Adequate edestin (Group III).....	6	9.2 $\pm$ 0.98	1.7	51 $\pm$ 3.2	55	14.6 $\pm$ 0.6	15.8	28.7
Adequate casein (Group IV).....	12	9.3 $\pm$ 0.64	2.4	51 $\pm$ 2.4	54	14.6 $\pm$ 1.2	15.5	28.6

\* Standard deviation.

Group II. Corpuscular hemoglobin, estimated according to the suggestion of Wintrobe (1929), was 13.5 gm.  $\times$  10<sup>-12</sup> and 11.2 gm.  $\times$  10<sup>-12</sup>, respectively. Again, of particular interest is the fact that although the cells varied in number, in size, and in the quantity of pigment present, the degree to which they were saturated with hemoglobin was strikingly constant (Table II). Evidently then, one may consider that the intrinsic composition of each erythrocyte remained the same in the groups of rats fed the

four specific rations of this experiment even though the gross picture of the blood was altered by certain dietary procedures. The constancy of this factor under unfavorable conditions has been noted by other investigators (Mayerson and Laurens, 1931). It is another remarkable example of the physiological adjustments that an organism can make to severe and abnormal dietary imposition.

Why the animals fed the ash-poor diet containing edestin should present upon examination a blood picture more nearly normal than that of the rats fed the low salt casein ration remains at present an unanswered question. It seems possible that the casein diet contained some unidentified factor, for instance phosphorus, that was absent in the edestin ration and which exerted a definite erythropoietic stimulus on the blood-forming organs.

A stimulation of reticulosis occurred in both groups of rats given the low salt rations (Table II). The phenomenon may have been somewhat more pronounced in the casein-fed animals. Orten and Smith (1934, b) have shown that an increased proportion of reticular erythrocytes appeared in rats maintained upon the deficient casein ration only after the concentration of hemoglobin had dropped to a submaximal level. The reticulocytosis observed in the present instance, in the two groups of rats on low salt rations is undoubtedly related to the drop in the concentration of heme pigment in each case. The proportion of reticulocytes is approximately the same as that noted by Orten and Smith at the particular level of hemoglobin, *i.e.* 13 gm., characteristic of our rats fed the deficient diets. On the basis of this correlation, the low salt edestin diet, in preventing polycythemia, appears to act by retarding the "passive accumulation of erythrocytes," the process largely responsible for the early characteristic increase in the number of erythrocytes caused by feeding the low ash casein diet (Orten and Smith, 1934, b).

*Water Metabolism*—The average daily intake of water and output of urine, week by week, of the four groups of rats grown upon the four modifications of the experimental diet are shown in Table III.

The average quantity of water consumed daily by the two groups of rats on the two adequate diets was 12.7 and 14.8 cc., respectively. It will be noted that while there was no orderly varia-



tion in these figures from week to week, the trend in the last weeks of the experiment was toward a higher consumption of water, no doubt related to the increased size of the animals. The rats on the low ash casein diet drank, on the average, a slightly larger quantity of water than did the two control groups. On the other hand, the rats fed the low salt food containing edestin drank

TABLE III

*Average Daily Intake of Water and Output of Urine, Week by Week, of Groups of Rats Grown upon Experimental Rations*

Time on experiment	Adequate casein			Adequate edestin			Low ash casein			Low ash edestin		
	No. of rats	H <sub>2</sub> O intake	Urine output	No. of rats	H <sub>2</sub> O intake	Urine output	No. of rats	H <sub>2</sub> O intake	Urine output	No. of rats	H <sub>2</sub> O intake	Urine output
<i>wks.</i>		<i>cc.</i>	<i>cc.</i>		<i>cc.</i>	<i>cc.</i>		<i>cc.</i>	<i>cc.</i>		<i>cc.</i>	<i>cc.</i>
1	6	11.3	2.2	6	13.0	2.9	6	13.3	2.0	6	13.4	3.7
2	6	14.0	1.9	6	14.3	2.5	6	16.4	2.5	6	12.6	3.5
3	6	13.6	2.1	6	13.3	2.3	6	14.1	3.9	6	16.5	4.3
4	6	11.6	1.6	6	13.8	2.6	6	13.9	3.1	6	16.6	4.4
5	6	11.4	2.4	6	15.0	3.4	11	12.9	4.5	9	19.4	6.0
6	6	14.0	2.8	6	14.8	3.2	11	13.7	3.5	12	19.0	8.0
7	7	11.5	2.4	6	16.3	3.1	11	14.9	6.8	12	19.6	8.5
8	7	10.4	2.3	6	15.6	3.6	11	13.8	4.8	17	19.8	9.0
9	7	11.9	1.9	6	14.7	3.6	11	14.7	5.0	12	22.0	9.9
10	7	13.3	2.2	6	14.4	3.9	11	14.1	5.3	12	21.1	9.4
11	7	13.1	2.5	6	16.4	4.2	10	11.7	4.4	12	21.3	12.9
12	7	15.9	2.3	6	14.9	3.4	10	14.1	4.9	11	21.5	10.9
13	7	13.3	2.3	6	15.4	3.6	10	14.9	5.0	10	21.3	12.1
Average per day...		12.7	2.2		14.8	3.3		14.0	4.3		18.8	7.9
Total for experimental period...		1157	202		1343	296		1277	390		1709	718

progressively more as the experiment progressed. For the last 5 weeks of the experiment, an average daily intake of 21 cc. of water was maintained in contrast to an average of 14 cc. for the other three groups.

Concomitant with the increased consumption of water of this group of rats was an augmented urinary excretion. The average

quantity collected in the last 3 weeks of the experiment was approximately 12 cc. per day. In contrast to the output of the other groups, this is a high figure. The diuretic effect of the low salt edestin ration definitely became more pronounced as the experiment progressed. The increased elimination of urine in certain individual rats was very marked, one rat actually excreting an average quantity of 30 cc. of urine every day for the last 2 weeks of the experiment.

The data show that the low salt casein food, although not as marked in its influence as the similar edestin diet, also seemed to exercise some diuretic effect. Even the ingestion of the adequate edestin ration causes some diuresis. However, it is the combination of the absence of salts and the protein, edestin, that most definitely affected urinary excretion in the albino rat.

#### CONCLUSIONS

On the basis of the data presented herein, the following conclusions seem pertinent:

The ingestion of a ration poor in salts containing edestin as the dietary protein does not induce as drastic changes in the blood stream of the rat as does a similar diet in which casein is the sole source of protein. The ration does not exert the specific erythropoietic effect of the casein diet. No polycythemia occurs; the erythrocytes are only slightly smaller in size than normal and the concentration of hemoglobin in each cell is not as markedly reduced.

However, the ash-free ration containing the protein, edestin, when fed to the rat, is conducive to the development of severe diuresis; whereas the comparable casein diet has only a slight influence. The diuresis is accompanied by polydipsia.

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## SOME ANALYSES OF SAMPLES OF BENCE-JONES PROTEIN

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The appearance of a peculiar protein in the urine of a person suffering from multiple myeloma was discovered by Bence-Jones in 1847. Since that time proteins having similar characteristics have often been recognized in urine and have been called Bence-Jones protein. Although hundreds of cases have been reported, very few detailed chemical or immunological studies have been made (1) to determine whether similar or identical proteins are being excreted either by the same individual from time to time or by different individuals. The most complete chemical analyses are those of Abderhalden and Rostoski (2) and of Hopkins and Savory (3). Some special determinations of chemical and physical properties have been made at times by other investigators, which include molecular weight by Svedberg and Sjögren (4), isoelectric point by Mainzer (5), solubility, specific rotation, ultra-violet absorption spectra, etc., by Hewitt (6). Careful immunological studies have been made by Hektoen (7), Bayne-Jones and Wilson (8), and by others. These and other investigations have led to the general conclusion that the substance of protein nature which appears in the urine of certain individuals and has the peculiar property of being insoluble in the faintly acid urine at 60°, yet redissoluble at the boiling point, is a protein chemically and immunologically distinct from the serum and tissue proteins. Some have also concluded that all Bence-Jones proteins are essentially identical in composition and are *chemically* indistinguishable, while Bayne-Jones and Wilson (8), from immunological studies, have concluded that they are not *biologically* identical.

The origin of these proteins is still obscure. Many theories of

their formation have been proposed: (1) products of the tumor cells themselves, (2) an abnormal substance produced by peculiar stimulation of the bone marrow cells, (3) an abnormal product of all tissue cells, (4) substances formed by abnormal digestive processes, and (5) changes in the serum proteins. The fourth of these theories was disproved by Folin and Denis (9) who showed that elimination continued at approximately the same rate during fasting as when nutrition was normal. The excretion in some cases of large quantities of protein and the presence of a small number of tumor cells leaves doubt as to the possibility of the first theory. With regard to the other three theories there is no decisive evidence in favor of any one.

This investigation is the beginning of a series we plan to make on Bence-Jones proteins as they become available. We plan first to show whether, from time to time, the individual excretes chemically identical compounds, as determined by modern methods, second whether those proteins excreted by different individuals are identical as determined by chemical analysis, and third, one of us (F.) plans to study these same proteins from the immunological point of view to supplement the few specific studies that have already been made. The frequency and seriousness of the disease multiple myeloma and the failure of early recognition of the condition justify extensive studies.

#### EXPERIMENTAL

An individual with Bence-Jones proteinuria was admitted to the hospital and 2 weeks later died of recognized multiple myeloma. Specimens of urine were collected over 24 hour periods and preserved with toluene. From two such specimens, one collected a week later than the other, samples of Bence-Jones proteins were prepared for chemical analysis by heating the urine to 60° at pH 5.5. The heavy flocculent precipitate was filtered, washed first with water previously heated to 60–65°, subsequently with dilute alcohol, then with absolute alcohol and ether, and finally dried in a vacuum desiccator over sulfuric acid for 48 hours.

Duplicate analyses were made in all cases on each sample. The moisture was determined by drying at 110° to constant weight, the ash by ignition in a platinum crucible, total nitrogen by the Pregl micro-Kjeldahl method, amino nitrogen by the Van Slyke method,

phosphorus by the Pregl micromethod as modified by Lieb and Wintersteiner (10), sulfur by a slight modification of the Denis method. The protein was hydrolyzed and the following determinations were made: amide nitrogen by adjusting the pH to phenol-

TABLE I

*Comparison of Analytical Values Obtained by Authors with Those of Other Investigators*

The nitrogen values are calculated in per cent of total nitrogen, while all other values are calculated in per cent of the ash- and moisture-free protein.

Constituent	Hopkins and Savory (3)	Abderhalden and Rostoski (2)	Authors	
			Sample I	Sample II
Ash.....			5.1	1.2
Moisture.....			10.6	4.8
Total N.....	16.2		18.0	18.1
Amino ".....			79.3	78.2
Phosphorus.....		(<0.01)	None	None
Sulfur.....			1.0	1.0
Amide N.....	1.3		5.6	5.7
Humin ".....			None	None
Tyrosine.....	4.2	1.7 (6.2)	6.8	6.7
Tryptophane.....	0.82	(1.7)	2.5	2.5
Cystine.....	0.57		3.0	3.0
Arginine.....	6.0		5.2	5.0
Histidine.....	1.2		1.1	1.2
Lysine.....	3.7		7.0	6.5
Glycine.....	Present	1.7		
Alanine.....	"	4.5		
Leucine.....	6.5	10.6		
Aspartic acid.....	2.1	4.5	4.5	4.7
Glutamic ".....	8.1	6.0	8.5	8.6
Phenylalanine.....	4.8	1.5		
Proline.....	2.7	1.9		
Valine.....	5.6			

The values in parentheses are taken from the data of Hewitt (6).

phthalein and aeration into standard acid, humin nitrogen by the Kjeldahl method, tyrosine and tryptophane by the method of Folin and Marenzi (11), cystine by the Folin and Marenzi method (12), arginine, histidine, and lysine by a method previously de-

scribed (13) with the modification for removal of cystine suggested by Vickery and Leavenworth (14); aspartic and glutamic acids were determined by the method of Jones and Moeller (15). The nitrogen values are expressed in per cent of the ash- and moisture-free protein. In Table I the values for certain constituents found by the two other groups of investigators have been recorded along with those of the authors.

#### DISCUSSION

It would have been interesting to have the values of Medes (16) for comparison but so far her findings have not been published in detail. There are, however, several points of interest in the comparison of our values with those of other investigators, which are remarkable when one considers that they are all isolation values made by the early methods. Our values for the amino acids are obtained by isolation also, with the exception of tyrosine, tryptophane, and cystine, in which cases we feel that the colorimetric methods are far superior. The total nitrogen value of Hopkins and Savory (3) is distinctly lower than ours, which may possibly be explained in part by their failure to correct for the ash and moisture content of the protein. The arginine value is higher than we have found. This may be because they considered the nitrogen in the filtrate from the histidine dichloride to be arginine nitrogen, which was added to their arginine value. It is doubtful that this is a correct assumption. Again it may be possible that we are dealing with proteins which are entirely different in chemical composition and in that case comparisons are of no analytical significance. The physical and chemical properties which have been accurately determined by several investigators, especially by Hewitt (6), seem to indicate that these proteins are chemically as well as biologically different. We do not feel, however, that the evidence is entirely convincing.

Since the origin of these proteins is unknown, it is interesting that some of the values we have found compare very closely with those reported for serum globulin. The tyrosine and cystine values are close to those reported by Reiner and Sobotka (17), while the arginine, histidine, and lysine are close to those of Block (18). However, the molecular weight reported by Svedberg and Sjögren (4) is just one-third of that of serum globulin. This

suggests the possibility that the serum globulin in this disease is split into three particles of equal size. Such a product would be an abnormal serum constituent and would be excreted by the kidney as is the case with other foreign proteins which enter the blood stream.

#### SUMMARY

Two samples of Bence-Jones protein were prepared from the urine collected at different periods from one individual and analyses made on each sample for ash, moisture, total nitrogen, amino nitrogen, phosphorus, sulfur, amide nitrogen, humin nitrogen, tyrosine, tryptophane, cystine, arginine, histidine, lysine, aspartic acid, and glutamic acid.

These values are reported in Table I along with those of some other investigators.

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## THE COMPOSITION OF TISSUES IN DEHYDRATION

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More than half the weight of the soft tissues of the body consists of water, and every marked and rapid loss of weight must, for the greater part, be a loss of water. We do not, however, speak of dehydration in such cases where the loss of weight is due to insufficient intake of food, although we know from the work of Gamble, Ross, and Tisdall (1) and others that in fasting body fluids are lost in considerable excess of the loss of body substance; nor do we then observe the characteristic symptoms of dehydration. When we speak of dehydration, it indicates our belief that in some cases the loss of water plays a specially important part in producing the clinical symptoms observed.

As body fluids are being lost both in dehydration and fasting, the question comes up, in what respect this loss of body fluids in dehydration differs from that in fasting. The purpose of the present work was to find the answer to this question. In this paper are presented the results of our analysis of the tissues of dehydrated animals.

The meaning of the term dehydration is somewhat uncertain. In the clinical case the factors involved are manifold: loss of fluids by vomiting and diarrhea, thirst and starvation by the inability to eat and drink, perhaps also increased water losses from the skin and lungs caused by fever and acidotic hyperpnea. Analyzing these factors, we find: dehydration in the strict sense of the word, by lack or loss of water; demineralization from loss of minerals with the gastric and intestinal secretions and insufficient intake of salts; starvation, that is, lack of organic and inorganic food materials and, perhaps, insufficient absorption due to diarrhea. In

order to study these factors separately we have attempted, as far as possible to produce pure dehydration; that is, lack of water but of nothing else. In future communications we hope to present analysis of tissues in complete fasting and in demineralization due to loss of intestinal secretions.

The earliest analysis of tissues in pure dehydration was published in 1854 by Falck and Scheffer (2) and one of the most important early contributions was made by Nothwang in Rubner's laboratory in 1892 (3). According to this latter work, pure dehydration leads to concentration, and fasting to dilution of the tissue fluids. In dehydration the water content was low, and the amount of ash high in relation to the water content. Quite the opposite was found in fasting; high water content and low ash content. As to the distribution of the water loss, it had already been found by Falck and Scheffer (2) that the most marked water loss occurred in the skin and the muscles. This was confirmed by Tobler (4), but, as his animals were dehydrated by diarrhea, they did not suffer from pure dehydration as we have defined it above. Schiff and coworkers (5, 6) found the most marked loss of water from the muscles; they did not analyze the skin.

These are the most important works on the composition of tissues in pure dehydration. Other papers bearing on the subject have been published by Landauer (7) on weight losses and survival periods of mice on a dry diet; by Kudo (8) on weight losses of various organs of rats on a dry diet; and by Garofeano and coworkers (9) on water content of various glandular organs in thirsting dogs.

### *Methods*

Eleven puppies, 27 to 55 days old, were used for the experiment. The five controls were fed dry milk powder mixed with water with the addition of cod liver oil and some ferric ammonium citrate; they were allowed to drink freely. The six dehydrated animals were given the same powder, mixed with a minimal amount of water, sufficient to allow the mixture to be forced through a stomach tube by a syringe. Vomiting was very rare. The experiments lasted from 2 to 7 days. The weight of the normal animals at the beginning of the experiment ranged from 597 to 3200 gm.,

that of the dehydrated animals from 1098 to 5000 gm. One of the controls was killed immediately; the others were kept from 5 to 9 days. One of them lost 31 gm.; the others gained from 155 to 239 gm. during the period of observation. The dehydrated animals were observed for a period of 2 to 7 days before they were killed. Their weight losses ranged from 176 to 450 gm. The animals were killed with ether, and as soon as they were unconscious the

TABLE I

*Concentration of Electrolytes, in Milli-Equivalents, per Liter of Tissue Water*  
Mean values, from five normal and six dehydrated animals.

		Chloride	Total base	Na	Base minus K	K
Muscle	Normal	53	203	70		93
	Dehydration	48	243	74		127
	<i>P</i> *	0.28	0.005	0.44		0.001
Skin	Normal	113	218		189	29
	Dehydration	139	289		252	37
	<i>P</i>	0.002	0.02		0.02	0.45
Brain	Normal	49	202	74		89
	Dehydration	62	232	91		100
	<i>P</i>	0.007	0.002	0.006		0.02
Liver	Normal	45	194	57		111
	Dehydration	61	230	76		126
	<i>P</i>	0.003	0.06	0.005		0.14
Kidney	Normal	82	241	105		78
	Dehydration	104	256	150		78
	<i>P</i>	0.04	0.12	0.001		

\* *P* indicates the probability that the difference is due to random variations of the individual values from which the means have been calculated. When *P* is 0.05 (a chance of 1 in 20) or less, we have assumed that the experimental procedure (dehydration), not random variations, is responsible for the difference.

collection of tissues began, three of us working as rapidly as possible to prevent losses from evaporation. The tissues were immediately put in weighing bottles. The samples were dried at 100° to constant weight, powdered, and analyzed. On the dried sample, fat was determined by the method described by Holt, Courtney, and Fales (10), nitrogen by the Kjeldahl method, chloride by the method of Laudet (11). Ashing was carried out in a muffle

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furnace at 400°. Total fixed base was determined on the ash solution by the method of Fiske (12), and potassium by the method of Fiske and Litarczek (13). Sodium was determined by the method of Barber and Kolthoff (14). Good checks were not always obtained in the determination of sodium, and we feel that the values for sodium are less accurate than those of the other tissue constituents. Especially was this true in the analysis of

TABLE II  
*Composition of Tissues in Dehydration*

Mean values, calculated from five normal and six dehydrated cases. Water, fat, and nitrogen are expressed in gm.; the other values, in milliequivalents per 100 gm. of fat-free dry substance.

		Water	Fat	N	Cl	Total base	Na	Base minus K	K
Muscle	Normal	499	34	13	28	102	35		46
	Dehydration	326	26	14	15	79	24		42
	P*	0.001	0.16	0.07	0.01	0.009	0.03		0.17
Skin	Normal	380	114	14	38	83		65	10
	Dehydration	217	132	14	30	63		56	8
	P	0.001	0.56		0.01	0.03		0.04	0.35
Brain	Normal	693	31	11	34	140	52		61
	Dehydration	599	27	11	38	140	54		61
	P	0.0006	0.39		0.17		0.57		
Liver	Normal	336	8	14	15	65	19		37
	Dehydration	294	24	14	18	68	24		37
	P	0.002	0.04		0.11	0.63	0.07		
Kidney	Normal	469	15	13	38	113	49		36
	Dehydration	427	16	13	44	109	60		34
	P	0.17			0.17	0.50	0.12		0.56

\* See foot-note to Table I.

the skin, where the sodium determinations were so unsatisfactory that they were discarded. In our discussion of the analysis of the skin we have used the values for non-potassium base, that is total fixed base minus potassium, in order to estimate approximately the changes in the sodium content.

In Tables I and II, to save space, we have given only the mean values. When the mean values obtained in the dehydrated animals were different from the mean normal values, we have calcu-

lated the probability ( $P$ ) that this difference is really significant and not due to individual variations.<sup>1</sup>

We have expressed our values either in gm. or in milli-equivalents per 100 gm. of fat-free dry substance. We were in some doubt whether this or the values for nitrogen were the best point of reference. The amount of nitrogen in the fat-free dry substance is, however, very constant, indicating a protein content of about 85 per cent, and we have found that it makes little difference

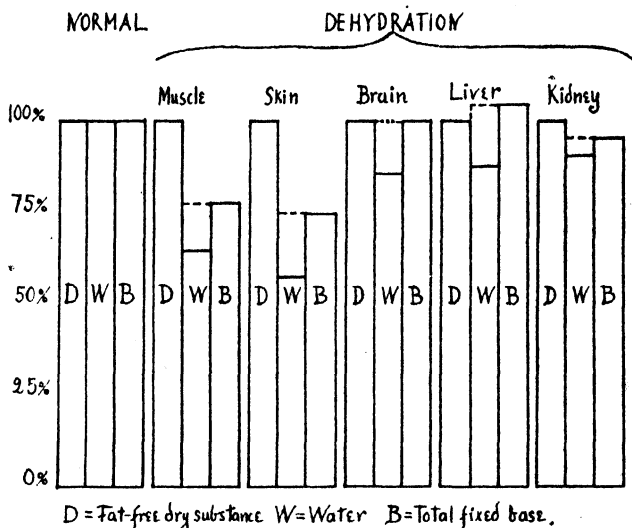


FIG. 1. Changes in water and total fixed base, expressed in per cent of normal values. The space between the top of the column for water and the dotted line indicates the degree of concentration of the tissue fluid.

whether fat-free dry substance or nitrogen is chosen as the point of reference. We have also calculated the electrolytes per liter of tissue water (Table I).

### Results

Table II shows the total changes that occurred in dehydration. Fig. 1 shows the changes expressed as per cent of normal values.

The changes that occurred were of two types: In muscles and

<sup>1</sup> The method used for calculating  $P$  is that given by Fisher (15).

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skin there was considerable loss of water and electrolytes (probably chiefly sodium and chloride), more of the former than the latter, resulting in a concentration of the tissue fluid. In liver and brain there was a small, but quite definite loss of water but no loss of electrolytes; the result was, of course, also in these organs a concentration of the tissue fluids. As to the kidneys, the statistical analysis shows that the values were too variable to allow any definite conclusions. In addition to these changes there was an increase of fat in the liver and some fatty infiltration or degeneration was seen in the histological specimen.

TABLE III

*Composition of Blood Serum Shortly before Death of Animals*

The non-protein nitrogen is expressed in mg. per 100 cc.; the other values, in milli-equivalents per liter.

	Dog No.	Total base	HCO <sub>3</sub>	Cl	Non-protein N
Normal	2		21	105	30
	6		19	108	23
	8	165	22	103	24
	13	173	21	106	32
Dehydration	3		22	131	72
	5		20	135	43
	9	177	23	119	33
	10	186	15	140	28
	12	196	24	129	39

In a few cases blood was obtained from the animals shortly before they were killed. The results of the analysis of the blood serum are given in Table III. The data are too few to allow definite conclusions, but there were two very high values for total fixed base and the chloride seems rather consistently to be higher than normal. Only in one case was there a definite decrease of the bicarbonate content.

### *Comment*

As the red blood corpuscles of the dog contain considerable amounts of sodium (16), it seems possible that this difference between the human and the dog may be present also in other body cells. Potassium, however, in the dog is probably present pre-

dominantly in the cells, as the potassium content of the blood plasma of the dog is about as low as in the human (16). In our cases, ranging the tissues according to their potassium content, we find that the liver has most potassium per liter of tissue water; then come muscles, brain, and kidney; the skin has a considerably lower potassium content than any other tissue. (The order might, possibly, be somewhat different if the kidneys could be analyzed free from urine.) On the whole, then, the more cellular organs contain more potassium than the organs poor in cells, and we think that the amount of potassium in the tissue water may be taken as a measure of the relationship between cellular and interstitial (including blood plasma) water in that tissue.

It is, therefore, of great interest that in no organ was there in dehydration any demonstrable loss of potassium in relation to fat-free dry substance. Potassium may have been lost, but, if so, only in proportion to the loss of fat-free dry substance. As pointed out by Gamble (17), the composition of cellular fluid in which the base is, chiefly, potassium, makes it unsuited to restore blood plasma volume, while the interstitial fluid is well adapted for that purpose. We think that the analysis of the tissues in our cases has again confirmed this prediction.

Interstitial and plasma fluid, on the other hand, has been lost in large amounts from the skin and the muscles, as indicated by the loss of water and base other than potassium.

Loss of tissue fluid could not have resulted in the increased concentration of base that was found in muscles, skin, and brain, and, probably, also in kidneys and liver. This finding indicates that the electrolytes were not eliminated quite as rapidly as water, and, as the electrolytes are eliminated chiefly through the kidneys, we have to assume that the kidneys could not rid the body of superfluous electrolytes. This breakdown of kidney function would, probably, not take place as long as interstitial water was still available for the maintenance of the blood plasma volume. In other words, finding, as in these experiments, an increased concentration of total fixed base per liter of tissue water suggests that the tissues have already been drained of most of their available interstitial fluid. The fluid losses ought, therefore, to give us some indication of the amounts of interstitial fluid available as a reserve store for plasma fluid. Per 100 gm. of fat-free dry substance, the



skin had suffered a loss of 43 per cent of its water, the muscles 35 per cent, the brain 14 per cent, the liver 13 per cent, and the kidneys, possibly, 9 per cent. In the brain, liver, and kidneys, the loss was not one of tissue fluids, but of water, as the decreased water content corresponds, approximately, to the increased concentration of base; this loss of water had, presumably, taken place after the kidney function had become impaired and the body was continuing to lose water while base was being held back. In the skin and the muscles, however, there had been a real loss of tissue fluid. The extent of this loss may be estimated from the loss of fixed base; in the muscles 1000 cc. of tissue water were, in dehydration, decreased to 654 cc. These 654 cc. contained 159 milli-equivalents of base which, under normal conditions (203 milli-equivalents per liter of muscle water) would have been contained in 783 cc. of water. The difference between this figure and the 654 cc. which actually remained in the muscles gives an approximate idea of the decrease of water volume due to concentration of the fluid. The total loss, per liter of water, of 346 cc. ( $1000 - 654$ ) would then consist of about 217 cc. ( $1000 - 783$ ) lost as tissue fluid and 129 cc. ( $346 - 217$ ) lost as water. In the skin we find by similar calculation 242 cc. lost as tissue fluid and 186 cc. as water. In round figures, therefore, the muscles and the skin had lost about 20 per cent of the tissue fluid present under normal conditions. It is to be understood that these figures are rough estimations and that the calculation does not take into account several factors such as the concentration and, possibly, increased base-binding power of the proteins, etc.

As the histological picture of our specimens makes it somewhat hard to understand where the large store of interstitial fluid is located in the muscles, it seems possible that the decreased volume of muscle water in dehydration was, at least to some extent, due to contraction of the muscle capillaries. The fluid thus lost would, of course, be chemically indistinguishable from interstitial fluid. In the skin, on the other hand, it is quite possible that the fluid lost to a major part consisted of true interstitial fluid.

#### SUMMARY

The results of these experiments demonstrate that uncomplicated dehydration, as produced simply by water deprivation,

leads to a concentration of electrolytes in the body fluids. The data obtained permit detailed description of the extent and source of the water and electrolyte losses which occur.

A loss of extracellular fluid was found to be a prominent feature and to derive, chiefly, from the muscle tissue, which lost 35 per cent of its water and 22 per cent of its base, and the skin, which lost 43 per cent of its water and 24 per cent of its base. The extracellular source of this loss is assumed from the fact that the base lost was not potassium. There was no demonstrable loss of potassium from any tissue analyzed, indicating that there was no withdrawal of intracellular water beyond the loss of water without electrolytes producing the observed rise in concentration.

In the brain, liver, and the kidneys the decrease in the volume of tissue water was slight and the water withdrawn was not accompanied by electrolyte loss. The only change, therefore, was an increase in concentration.

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## THE SOLUBILITY OF THE PLASMA PROTEINS

### II. DEPENDENCE ON pH, TEMPERATURE, AND LIPID CONTENT IN CONCENTRATED SOLUTIONS OF POTASSIUM PHOS- PHATE AND APPLICATION TO THEIR SEPARATE PRECIPITATION

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In a paper from this laboratory, describing the dependence of the solubility of the plasma proteins in concentrated salt solutions on the salt and plasma concentrations (1), we pointed out the advantage of using potassium phosphate as a precipitating salt inasmuch as the salt concentration and pH of such solutions could be varied independently as described by Cohn (2).

For the specific purpose of ascertaining the optimum conditions for separating the plasma proteins by single fractional precipitation, we have continued the study of the solubilities of these proteins in concentrated solutions of potassium phosphate and in this paper report data on the effect of pH, temperature, and removal of lipids on the solubility curve of the proteins of horse plasma. Though the dependence of the solubility of protein in salt solutions on pH is clearly established (3, 4), but few data on the quantitative relation between the solubility of the serum proteins in concentrated salt solutions and pH have been presented over the pH range encountered in the salt solutions commonly used for salting-out purposes. Csapó and von Klobusitzky (5) observed but little effect in the precipitating power of sodium sulfate and sodium chloride solutions on the serum proteins when the pH changed from 4.8 to 7.4. Wu (6), in studying the solubility of serum proteins in sodium sulfate solutions, observed no appreciable effect of pH on their solubility over a pH range of from 5.0 to 9. Howe

(7) observed that a greater concentration of phosphate in solutions of mono- and disodium phosphate or mono- and dipotassium phosphate was required to produce equal precipitation of the plasma proteins as the pH of the solutions fell from 8.0 to 5.8, a behavior that might be considered without further analysis contrary to the usually observed decrease in protein solubility as the isoelectric point is approached.

Wu (6) reported a decrease in solubility of the lipid-free serum as compared to the natural serum. His curves, however, are not detailed enough to permit an accurate analysis of the individual protein fractions, particularly the globulin portion, where changes in the curve might be expected since there is evidence that euglobulin is a complex of pseudoglobulin and lipids (8, 9). An analysis of the individual fractions of a lipid-free serum, whose solubility is different from that of natural serum, by such specified salt concentrations as given by Howe (10), may give solubility data that have but little relation to the solubility of the individual serum proteins.

Mellanby (11) observed that ammonium sulfate was slightly more effective as a precipitant of the serum proteins at 0° than at 40°.

#### EXPERIMENTAL

*Effect of pH*—Fig. 1 presents the solubility curves of the proteins of horse plasma in potassium phosphate solutions of pH 5.4, 6.5, and 7.7,<sup>1</sup> determined as described in the previous paper (1). All

<sup>1</sup> The pH of 5.4 was selected since it was thought that a curve at the isoelectric point of serum globulin might increase the difference in the solubilities of the globulin and albumin fractions. Moreover, this is as acid a pH as can be obtained with a buffer solution of mono- and dibasic potassium phosphates. The pH of 7.7 is approximately the alkaline limit of such a buffer solution. And the pH of 6.5 is a convenient intermediate point. The solutions of varying ionic strength of pH 6.5 were made by dilution of a 3 M solution of phosphate consisting of equimolecular amounts of the dibasic and monobasic potassium salts as described in the paper referred to above (1). The solutions of pH 5.4 and 7.7 required individually constructed solutions for each concentration, since the mole fraction of  $K_2HPO_4$  which provides a constant pH at either of these pH values varies with change in the salt concentration of the solution. The mole fraction of  $K_2HPO_4$  required to give the pH of 5.4 or 7.7 at any desired phosphate concentration was taken from the interpolation tables and graphs of Green (12), the values beyond the concentrations given in that paper being deter-

three curves were made from a single sample of horse plasma collected aseptically. The curve at pH 5.4 was determined the day the plasma was drawn. The plasma was then kept under sterile conditions at 10°, and the curves at pH 6.5 and 7.7 were determined respectively 6 and 15 days after the plasma was collected. Under such conditions the solubility of the proteins remains unaltered. Moreover, the curves at the two latter pH values agree with others obtained at these pH values with fresh plasma.

The ordinate on the left represents the solubility of protein nitrogen expressed as gm. of nitrogen per liter of filtrate. The ordinate on the right represents the logarithm of the solubility. The abscissa represents the ionic strength of the potassium phosphate per liter of the plasma phosphate solutions,<sup>2</sup> the solutions consisting of 1 cc. of serum to 30 cc. of the various phosphate solutions. The solubility curve at each pH is represented by the solid line, the logarithm curve by the broken line. This latter curve was used to determine the points of break in the solubility curve as described in our previous paper (1). Since no consistent break occurs at the ionic strengths corresponding to 2.50 at pH 6.5 (molar concentration of 1.25), the curves are drawn without any indication of the precipitation of euglobulin. The curve at pH 5.4 could not be extended further because of the limited solubility of potassium phosphate at that pH.

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mined by extrapolation. The desired mole fraction and concentration of each solution at pH 5.4 and 7.7 were obtained by weighing out the required amounts of the dried dibasic and monobasic salts or by titrating from standardized solutions of  $K_2HPO_4$  and  $H_2PO_4$ , the calculated number of cc. of each solution. The solutions were then checked for total phosphate by the method of Fiske and Subbarow (13) and for approximate pH colorimetrically. The determinations were carried out at 39° in order to increase the solubility of the phosphate in the most acid solutions.

Although the effect of temperature on pH in such concentrated phosphate buffers has not been determined, there is no appreciable change in dissociation constant or activity coefficient with temperature at the concentrations studied (14).

<sup>2</sup> Ionic strength per liter of solution is used as a matter of convenience. It may be defined as  $\Gamma/2$  where  $\Gamma = \sum CZ^2$ , as used by Debye and Hückel (15),  $C$  being the stoichiometrical molarity of each ion and  $Z$  its valence. This should be differentiated from ionic strength designated by Lewis and Randall's  $\mu$  (16), where the concentration of each ion is expressed as stoichiometrical molality.

The solubilities of all the fractions increase with increasing pH. Though no two entire curves are superimposable, the corresponding fractions of each of the three curves are very nearly superimposable separately. In the terms of Cohn's equation (17) which defines the solubility of a protein in concentrated salt solutions

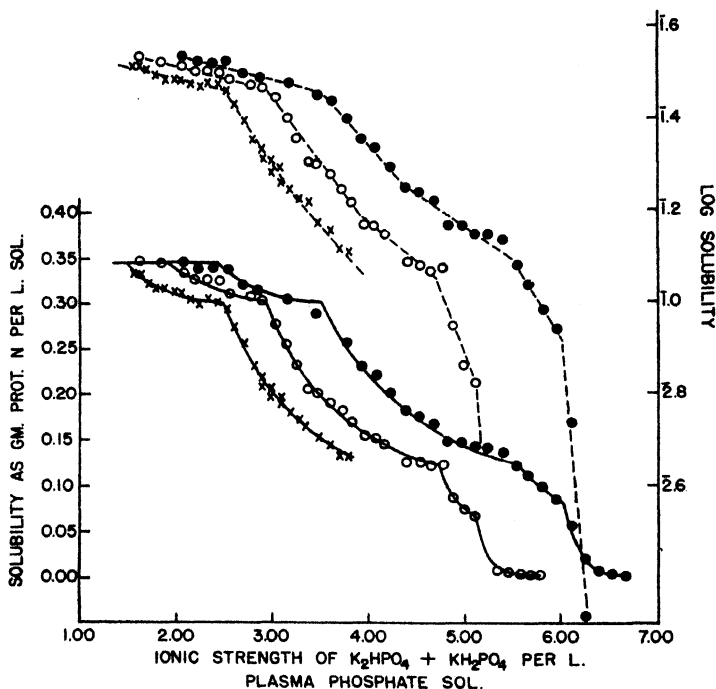


FIG. 1. Solubility curves of horse plasma proteins at 39°.  $\times$  represents the solubility at pH 5.4;  $\circ$ , at pH 6.5;  $\bullet$ , at pH 7.7.

as  $\log S = \beta - K_s \frac{\Gamma}{2}$ , where  $\beta$  is an intercept constant,  $K_s$  a constant representing the slope of the curve, and  $\frac{\Gamma}{2}$  the salt concentration in terms of ionic strength per liter of solution,<sup>2</sup> the data of Fig. 1 indicate that the  $K_s$  constants or slopes of the different fractions are affected but slightly by pH, whereas the  $\beta$  constants are dependent upon pH and for each fraction are affected differently.

In an attempt to obtain more quantitative data on the relationship between pH and the constants  $K_s$  and  $\beta$ , the logarithms of the solubilities of the globulin, probably pseudoglobulin, fraction of the curves were plotted against salt concentration, the zero solubility of this fraction on the curves being made by logarithmic extension as 0.09 mg. of nitrogen per liter (see (1) p. 183). The numerical values for  $K_s$  and  $\beta$  so obtained at the three pH values are given in Table I.

*Effect of Temperature*—Fig. 2 presents the solubility curves of the proteins of horse plasma at 1°, 20°, and 39° determined on samples from the same horse plasma in concentrated potassium

TABLE I  
*K<sub>s</sub> and  $\beta$  Constants for "Pseudoglobulin" Fraction from Experimental Data of Figs. 1 to 3*

Fig. No.	Source of sample	Total N concentration	pH	Temperature	$K_s$	$\beta$
		gm. per l.*		°C.		
1	Horse plasma	0.345	5.4	39	0.52	0.61
	" "	0.345	6.5	39	0.51	0.82
	" "	0.345	7.7	39	0.48	1.06
2	" "	0.325	6.5	1	0.47	0.81
	" "	0.315	6.5	20	0.49	0.70
	" "	0.320	6.5	39	0.49	0.70
3	Serum powder	0.310	6.5	20	0.49	0.68
	" "	0.620	6.5	20	0.49	0.90

\* Total nitrogen concentration per liter where no precipitation occurred.

phosphate solutions of pH 6.5. The curve at 20° was determined the day the plasma was drawn. The curves at 1° and 39° were determined 24 hours later, the serum having been kept under aseptic conditions at 10° during the interim.

The presence of a euglobulin break is equivocal, but the curves have been drawn so as to reflect a differential precipitation over the euglobulin range. The three heavy crosses represent the solubility of nitrogen in the filtrate from sodium sulfate solutions at 37.5° according to the method of Howe (10). The ionic strengths of 4.44, 4.56, and 4.87 correspond respectively to 21.0, 21.5, and 23.0 gm. per cent of sodium sulfate.



The solubilities in the phosphate solutions at 20° and 39° are practically identical, while the solubility at a given salt concentration is slightly greater at 1° than at the two higher temperatures.

The  $K_s$  constants of the different fractions appear to be independent of temperature over this range. The  $\beta$  constant of a given fraction apparently increases when the temperature approaches 0°. The numerical values for  $K_s$  and  $\beta$  for the pseudoglobulin fraction at each temperature were estimated and are given in Table I.

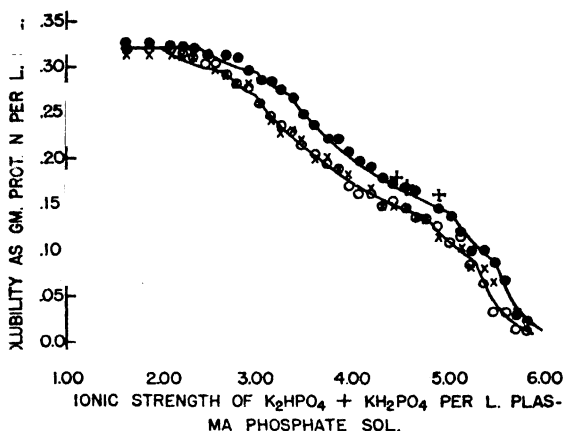


FIG. 2. Solubility curves of horse plasma proteins. ● represents the solubility at 1°; X, at 20°; ○, at 39°; ✕, in sodium sulfate at 37.5°.

*Effect of Lipids*—Fig. 3 presents the solubility curve of lipid-free horse serum proteins at pH 6.5 and a temperature of 20°. Lipid-free serum protein powder was prepared according to the method of Hewitt (18). These proteins were readily soluble in 0.7 gm. per cent saline, forming a clear solution with a protein concentration as high as 25 gm. per cent.<sup>3</sup> The composition of the lipid-free serum protein solution, which was added to the various phosphate solutions, was: serum protein 6.1 gm. per cent, non-protein nitrogen 6 mg. per cent, sodium 137 milli-equivalents per liter, chloride

<sup>3</sup> In a few instances the antitoxic titer of antidiphtheria horse serum, as measured by skin tests on guinea pigs, was correspondingly raised; i.e., to 5 to 10 times the titer of the original horse serum.

116 milli-equivalents per liter, calcium 12 mg. per cent, and cholesterol 0.

The upper curve represents the solubilities from an experiment in which 1 cc. portions of lipid-free serum protein solution were added to 15 cc. of the phosphate solutions; *i.e.*, a dilution of the

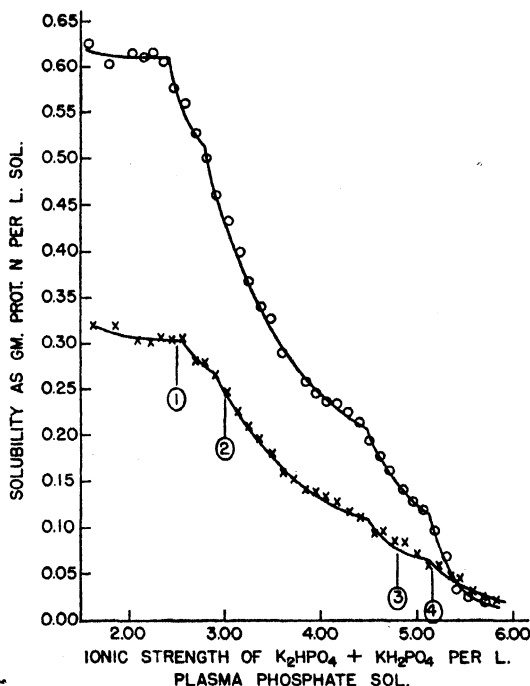


FIG. 3. Solubility curves of lipid-free horse serum proteins at 20°.  $\times$  represents the solubility at a dilution of lipid-free serum solution in phosphate solution of 1:31;  $\circ$ , of 1:16. The points 1, 2, 3, and 4 represent the phosphate concentrations at which breaks occur in the precipitation curves of natural lipid-containing horse plasma.

serum protein solution of 1:16 instead of the 1:31 dilution used in the majority of our experiments. The lower curve represents the solubilities of the same lipid-free serum protein solution diluted 1:31 in the phosphate solutions.

The character of the curve is changed but little from that of

fresh horse serum. From the data of the lower curve at the usual dilution there is a questionably significant precipitation between the ionic strengths of 1.55 and 2.60 as might be expected for serum from which fibrinogen has been withdrawn as fibrin. Between the ionic strengths of 2.60 and 2.90 there is a precipitation which may be attributed to euglobulin. The major portion of the globulin is precipitated between the ionic strengths of 2.90 and 4.40. The albumin begins to precipitate at an ionic strength of 4.50 instead of at that of 4.80 as observed with natural serum, thus indicating a slight decrease in solubility in this protein fraction. The break in the albumin portion of the curve occurs at an ionic strength of 5.15 and corresponds to that with natural serum.

Without a correction for the precipitation of euglobulin with pseudoglobulin (1) the  $K_s$  and  $\beta$  constants of the second globulin fraction (pseudoglobulin) have been estimated and are given in Table I. The numerical values of the  $K_s$  and  $\beta$  constants are in close agreement with those obtained with pseudoglobulin of natural plasma at the same pH and similar protein concentration. The increase in protein nitrogen solubility with increase in serum concentration is shown by the  $K_s$  and  $\beta$  constants for pseudoglobulin (Table I) to be due to an increase in the  $\beta$  constant, thus confirming the relationship suggested in our previous paper (1).

#### DISCUSSION

The increase in solubility of the plasma proteins over the globulin and albumin portions of the curves with increase in pH from 5.4 to 7.7 as shown in Fig. 1, where the salt concentration is expressed as ionic strength, conforms to the expected behavior of proteins whose isoelectric points are 5.4 and 4.9. The increase in solubility of the fibrinogen portion over the same increase in pH indicates an isoelectric point of fibrinogen below 5.4 and agrees with Florkin's (19) evidence on this point. Our data are in agreement with Howe's findings in the experiments with phosphate solutions already mentioned (7). For, if our salt concentrations are plotted as molar concentrations, the solubility of nitrogen in the filtrate of a given molar concentration decreases as the pH rises from 5.4 to 7.7. Conversely, if Howe's phosphate concentrations are expressed in terms of ionic strength, the solubility of the plasma proteins increases as the pH rises from 5.8 to 8.0. The conformity

of the data when salt concentrations are expressed as ionic strengths with the expected behavior indicates the appropriateness of using this unit of concentration when expressing the solubility of proteins as a function of salt concentrations. It is for this reason that we have used in this paper units of ionic strength rather than molarity in expressing the concentrations of the phosphate solutions.

Our finding that the  $K_s$  constants of the various fractions are affected but little by pH or temperature, while the  $\beta$  constants are dependent upon both these factors, agrees with the findings of Florkin for fibrinogen (19) and Green for carboxyhemoglobin (20). A slight decrease in  $K_s$  with increasing pH, as shown in Fig. 1 (see Table I) may be experimental error, but may be due to the specific ion effects of monovalent and divalent phosphate ions. However, evidence from the effect of pH on the solubility of fibrinogen in sulfate and phosphate solutions (18) indicates that the specific ion effect of these two ions would account for only a small fraction of the observed change in solubility and that we are justified in assuming that the greater part of the solubility change is due to a relationship between pH and  $\beta$ .

The numerical values of the  $K_s$  and  $\beta$  constants over the globulin or pseudoglobulin portions of the curves are presented for the purpose of comparing and characterizing changes in solubilities rather than for the purpose of obtaining accurate numerical values for these constants. However, the order of magnitude of the  $K_s$  obtained in our experiments, approximately 0.50, may have further significance when it can be compared with the  $K_s$  of purified horse pseudoglobulin in phosphate solutions. At the present time comparison can be made only with the  $K_s$  of 1.47, calculated from Sørensen's data<sup>4</sup> on purified horse pseudoglobulin in concentrated solutions of ammonium sulfate. Though these  $K_s$  values are not directly comparable, the difference seems more than is to be expected from specific ion effects, since we have obtained pseudoglobulin  $K_s$  values of approximately 0.50 from solubility curves in sodium sulfate solutions and Florkin for fibrinogen (19) and Green for carboxyhemoglobin (20) have observed lesser  $K_s$  values in sulfate solutions than in phosphate solutions. Such an increase in the  $K_s$  of purified pseudoglobulin above that obtained for the

<sup>4</sup> Taken from Cohn (21).

native pseudoglobulin of plasma again raises the question of whether procedures of purification alter the protein molecular complex.

*Estimation of Individual Plasma Proteins from Single Fractional Precipitation with Potassium Phosphate Solutions*

The results presented in Fig. 1 indicate that the effect of pH on the solubility of the plasma proteins over the pH range from 5.4 to 7.7 may be large enough to introduce significant errors in salting-out procedures as commonly used today, for commercial preparations of ammonium sulfate and sodium sulfate are notoriously variable in the pH of their solutions.<sup>5</sup> Furthermore, the pH of a solution of these unbuffered salts and plasma may depend not only upon the pH of the salt solution but also on the dilution of the plasma, since this may affect the pH of the plasma salt solution. The data presented in Fig. 1 therefore suggest the advisability of using effectively buffered solutions in procedures of fractional precipitation where a particular salt concentration is specified.

Fig. 2 shows that ordinary variations of room temperature do not affect the solubility of the plasma proteins in these phosphate solutions beyond the limit of experimental error. In this respect potassium phosphate solutions as the precipitating reagent in salting-out procedures provide an advantage over solutions of sodium sulfate whose temperature must be maintained at approximately 37.5°.

Since the maximum buffer effect of potassium phosphate is obtained at a pH of 6.5 and since concentrations of this salt at this pH may be made by dilution of a 3 M solution together with the fact that no improvement in the separation of the protein fractions results at pH 5.4 or 7.7, available data suggest the use of potassium phosphate solutions of pH 6.5 in the precipitation of individual plasma proteins.

<sup>5</sup> We have found that solutions of c.p. ammonium sulfate in distilled water vary from an approximate pH of 4.5 to 5.5. Some preparations even after recrystallization have been so acid as to cause complete precipitation of the serum proteins when 0.5 cc. of serum was added to 7.25 cc. of water and 7.75 cc. of saturated ammonium sulfate solution. Dr. Scriver and Dr. Berglund (personal communication) found such variations in the pH of sodium sulfate solutions that they felt it was necessary to buffer their sodium sulfate solutions for use as a precipitating reagent.

In our previous paper (1) we give the reasons for believing that at pH 6.5 the first portion of the curve from 0.8 to 1.25 M<sup>6</sup> represents the precipitation of fibrinogen, while the second portion from 1.25 to 1.50 M in the horse plasma curves of that paper reflects the precipitation of euglobulin. For human plasmas no differential precipitation of euglobulin and pseudoglobulin was observed, and this observation has been confirmed by Kydd (22). With the accumulation of further data many horse plasmas fail to show any differential precipitation of two globulin fractions, and the question arises as to whether some of the euglobulin is precipitated with the fibrinogen or is present in such small amounts as to escape detection. Because of the uncertainty as to whether the first fraction is entirely fibrinogen and our knowledge that no other portion of the curve represents the complete precipitation of a pure fraction, it would be safer to refer to the portions of the curves as Fractions I, II, III, IV, etc. But where a fraction over a particular salt range does not appear such as in the curves of Fig. 1, a numerical nomenclature of the fractions might be confusing. Therefore, though aware of the uncertainty, we may for convenience refer to the portions of the curves as representing the fibrinogen, euglobulin, pseudoglobulin, Albumin 1, and Albumin 2 fractions. The finding of a break in the albumin portion of the curve indicative of two albumin fractions has been repeatedly confirmed.

With potassium phosphate solutions of pH 6.5, the single fractional precipitation of individual protein fractions at specific concentrations may be summarized as follows:

Fraction I, containing fibrinogen, is precipitated at a molar concentration of phosphate of 1.25.<sup>6</sup>

Fraction II, probably largely euglobulin, but no more than two-thirds of the total amount, begins to precipitate at a molar concentration of phosphate of 1.25 and continues to be precipitated without the active precipitation of pseudoglobulin up to a molar concentration of 1.50.

Fraction III, representing approximately 80 per cent of the pseudoglobulin and 30 per cent of the euglobulin, precipitates between the molar concentrations of 1.50 and 2.40.

<sup>6</sup> At pH 6.5 the ionic strength of potassium phosphate per liter of solution =  $2 \times$  molar concentration of phosphate.

Fraction IV, consisting of Albumin 1 with some pseudoglobulin, begins to precipitate at a molar concentration of phosphate of 2.40 and continues to be precipitated without the active precipitation of Albumin 2 up to a molar concentration of 2.58.

Fraction V, consisting largely of Albumin 2 with a trace of pseudoglobulin and Albumin 1, begins to precipitate at a molar concentration of phosphate of 2.58 and is completely precipitated at a 3.00 M concentration of phosphate.

From the nature of the precipitation curves it is clear that an estimation of the euglobulin fraction by single fractional precipitation in potassium phosphate solution over a pH range from 5.4 to 7.7 is subject to such an error as to make a quantitative estimation of euglobulin concentration unwarranted. Complete precipitation curves of plasma in sodium sulfate solutions at 37.5° according to the method of Howe (10) indicate that an error of the same order of magnitude applies to euglobulin determinations by that method.

The inability to make a quantitative estimation of certain of the plasma proteins by single fractional precipitation does not mean that they cannot be separated by repeated fractional precipitation.

#### SUMMARY

The effect of pH, temperature, and removal of lipids on the solubility of the plasma or serum proteins in concentrated solutions of potassium phosphate is described. Neither the variations of pH or temperature over the ranges studied nor the removal of lipids affects the completeness of separation of these proteins by single fractional precipitation.

The significance of the observed dependence of the solubility of the plasma proteins on pH in the determination of plasma protein fractions by the commonly used salting-out procedures is discussed.

The concentrations of potassium phosphate in plasma phosphate solutions of pH 6.5 that may be used in estimating the concentration of certain plasma protein fractions or in accomplishing their separation by repeated fractional precipitation are given.

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## THE ABSORPTION AND METABOLISM OF CYSTINE AND OF CERTAIN CYSTINE DERIVATIVES IN DOGS WITH ISOLATED INTESTINAL LOOPS

BY JAMES C. ANDREWS, CHARLES G. JOHNSTON, AND  
KATHLEEN CRANDALL ANDREWS

*(From the Departments of Physiological Chemistry and Research Surgery,  
School of Medicine, University of Pennsylvania, Philadelphia)*

Further studies have been made of the rate of absorption of *l*-cystine, both in suspension and as the sodium salt. For this series of determinations the same dog has been used throughout with the following results. (1) The quantity of cystine absorbed from a given loop, the amount introduced and time of absorption being the same, is independent of whether the cystine is administered as the very soluble sodium salt or as a suspension of isoelectric cystine. (2) The quantity of cystine absorbed per hour is not constant but increases the longer the cystine is left in the loop. (3) The quantity of cystine absorbed increases as the amount introduced (as sodium salt or isoelectric cystine) is increased. Quadrupling the size of the original charge more than doubles the amount absorbed.

The relative rates of absorption have been determined for the following compounds (arranged in order of speed of absorption): cysteic acid > cysteic acid hydantoin > cystine phenylhydantoin > dibenzoylcystine > cystine > cystine hydantoin.

Metabolic experiments on the oxidation of the above compounds after absorption from isolated loops have confirmed the results of previous investigators in the case of cystine, cysteic acid, cystine phenylhydantoin, and dibenzoylcystine.

Cysteic acid hydantoin is excreted about 75 per cent as unoxidized sulfur, while the increase in sulfate accounts for 15 to 20 per cent of the sulfur absorbed. The sulfur of cystine hydantoin is excreted unoxidized.



**COMPOSITION OF WHOLE SOUND TEETH, ENAMEL, AND DENTIN**

By W. D. ARMSTRONG

*(From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis)*

The composition of the inorganic phase of whole teeth varies with the proportions of enamel and dentin. The mineral composition of enamel varies in teeth taken from one mouth as much as that obtained from teeth taken from different mouths. The composition of dentin, whether obtained from the same mouth or different mouths, is very constant. The magnesium content of dentin is twice as high as that of enamel. The  $\text{Ca}_3(\text{PO}_4)_2:\text{CaCO}_3$  ratio of enamel is twice that of dentin. The relation of composition to susceptibility to decay and age of eruption has been investigated.

**THE OXIDATION-REDUCTION POTENTIALS OF CYANIDE-HEMOCHROMOGEN**

By E. S. GUZMAN BARRON AND A. BAIRD HASTINGS

*(From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago)*

It is known that hemin derivatives form the active nucleus of a number of enzymes concerned with biological oxidations. Since it is possible to predict whether an oxidative catalyst will produce the oxidation of an oxidizable substrate from a knowledge of their free energies, we are now engaged in a comprehensive study of the oxidation-reduction potentials of hemin and hemochromogens. Cyanide-hemochromogen forms a perfectly reversible oxidation-reduction system where the experimental values found agree with the calculated values according to the equation,

$$E_A = E'_0 - \frac{RT}{F} \ln \frac{[\text{reduced cyanide-hemochromogen}]}{[\text{oxidized cyanide-hemochromogen}]}$$

The  $E'_0$  of this system, measured at  $30^\circ$  in the presence of an excess of cyanide to prevent dissociation of the hemochromogen, has been found to be  $-0.183$  volt, a value which is independent of the pH of the solution within the pH range 9.89 to 13.0. Below pH 9.89 it is difficult to keep the hemochromogen from dissociating, since a large part of the cyanide is present as undissociated HCN which does not give a hemochromogen; and the potentials thus

obtained depend on the equilibrium values of hemin and hemochromogen. Although hydrogen in the presence of colloidal palladium quickly reduces hemin and nicotine-hemochromogen, it does not reduce oxidized cyanide-hemochromogen, even after 3 hours. Titrations were therefore performed by reducing the oxidized hemochromogen either with hydrosulfite or titanous borate-tartrate. The reoxidation of reduced cyanide-hemochromogen by atmospheric oxygen bubbling at a rate of 2 cc. per minute, at 20°, is performed in 4 minutes, 35 seconds, rather a slow rate when compared with the rate of reoxidation of reduced nicotine-hemochromogen, which is 2 minutes, 40 seconds. The significance of this is discussed.

#### **TRYPTOPHANE METABOLISM OF THE DIPHTHERIA BACILLUS**

By LYLE C. BAUGUESS

*(From the Biochemical Laboratory, State University of Iowa, Iowa City)*

Mueller, Klise, Porter, and Graybiel have shown that growth of the strain of diphtheria bacillus isolated by Yü is enhanced by *l*-tryptophane. We have studied the growth of this organism on *d*- and *dl*-tryptophane and related indole derivatives.

Each of these compounds was incorporated in a tryptophane-deficient, but otherwise adequate, medium in amounts molecularly equivalent to 1 mg. of tryptophane per 10 ml. After sterilization each culture was inoculated with the organism and incubated at 38°. The containers were shaken at 12 hour intervals. After 48 hours incubation, growth was estimated nephelometrically.

*d*-, *dl*-, and *l*-tryptophane were found equally efficient in the promotion of growth. Of the indole derivatives tested ( $\beta$ -3-indoleacrylic acid,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid, *l*- and *dl*-indolelactic acids, and  $\beta$ -3-indolepyruvic acid), the *d* component of *dl*-indolelactic acid and  $\beta$ -3-indolepyruvic acid promoted as good growth as tryptophane. The other derivatives were quite ineffective as growth promoters.

#### **A POLYHYDROXY ACID FROM THE SWEET PEPPER**

By EMIL J. BAUMANN, DAVID B. SPRINSON, AND  
NANNETTE METZGER

*(From the Laboratory Division, Montefiore Hospital, New York)*

A polyhydroxy monobasic acid has been separated from the sweet green pepper (*Capsicum annuum*) which crystallizes from

water in prisms. It is slightly soluble in cold water, acetone, and methylethyl ketone and more soluble in methyl alcohol. Preliminary experiments indicate that it contains a carboxyl, two methoxyl, and two or three hydroxyl groups, that it is a saturated substance containing no sulfur, halogens, or phosphorus, and that its empirical formula is  $C_8H_{14}O_8$ .

R. J. Williams and his collaborators have reported experiments which show that one of the constituents of bios is a polyhydroxy saturated acid which has a molecular weight of 150 to 200. The hydroxy acid from peppers, however, stimulates yeast growth only slightly as compared to that reported by Williams for concentrates containing the pantothenic acid fraction.

#### THE OXIDATION OF CERTAIN AMINO ACIDS BY "RESTING" BACILLUS PROTEUS

By FREDERICK BERNHEIM, MARY L. C. BERNHEIM, AND  
M. DOROTHY WEBSTER

(From the Departments of Physiology and Biochemistry, Duke University  
School of Medicine, Durham, North Carolina)

The various amino acids are oxidized to different extents and at different rates by "resting" *Bacillus proteus* in 0.05 M phosphate buffer at pH 7.8. Leucine, phenylalanine, and methionine are oxidized most rapidly and utilize only 1 atom of oxygen per molecule. Serine, alanine, and proline are next and utilize 3, 4, and 5 atoms of oxygen respectively. Tyrosine and tryptophane are oxidized more slowly, with the utilization of 2 and 3 atoms of oxygen respectively. Valine, isoleucine, oxypoline, and histidine are oxidized very slowly and no definite oxygen uptakes could be measured. The values for the oxygen uptakes are independent within limits of the amount of *proteus* present. Deamination occurs in all cases and  $CO_2$  is produced only with serine, alanine, tryptophane, and proline. The relative rates of methylene blue reduction are different from the oxidation rates. Beginning with the most rapid the order is as follows: proline, serine, isoleucine, leucine, valine, histidine, oxypoline, alanine, phenylalanine, tryptophane, methionine, and tyrosine. Only the natural optical isomers are attacked. 0.005 M KCN completely inhibits the oxidation of all these amino acids.

**A TAXONOMIC STUDY OF THE DISTRIBUTION OF VITAMINS A AND D IN ONE HUNDRED SPECIES OF FISH**

BY CHARLES E. BILLS, FRANCIS G. McDONALD, O. N. MASSENGALE, MIRIAM IMBODEN, HELEN HALL, W. D. HERGERT, AND J. C. WALLENMEYER

*(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana)*

Assays for vitamins A and D were made on the liver or body oils of 100 species, representing seventeen zoological orders. Liver oils were more potent than body oils. For a given species, there was no predictable relationship between the vitamin A and D potencies. In general, but with many exceptions, liver oils rich in one vitamin tend to be rich in the other, and the potency in both tends to vary inversely with the oil content of the liver. The theory that the soft boned species contain but little vitamin D was confirmed. Nevertheless, the only species whose livers yielded no measurable amount of vitamin D were the gray sole and the sturgeon. The basking shark, despite its surface feeding, showed no vitamin A in its liver oil, and very little vitamin D.

The taxonomic study revealed that the highest concentrations of both vitamins were in species of the order Percomorphi (mackerels, tunas, sea-basses, swordfish). A new form of vitamin D was discovered in the bluefin tuna. Next below the percomorphs in potency came the species of the order Cataphracti (rockfishes, sculpins, etc.). The Heterosomata (halibut and other flatfishes) predominated in vitamin A; the Holconoti (viviparous perches) in vitamin D. The lower orders were generally poor in vitamin D. Three-quarters of all the liver oils was more potent than cod liver oil in vitamin D, and nearly all surpassed it in vitamin A. Numerous percomorph oils were 100 to 400 times more potent than cod liver oil in either or both vitamins.

**HYPERSIDEREMIA FOLLOWING THE ORAL ADMINISTRATION OF IRON\***

BY FRANKLIN C. BING, RAMON F. HANZAL, AND  
VICTOR C. MYERS

(From the Department of Biochemistry and the Institute of Pathology, School of Medicine, Western Reserve University, Cleveland)

The absorption of iron from the alimentary tract was studied in dogs by estimation of the non-hemoglobin iron of the blood serum. Two methods of analysis were used. It was observed that the total iron of the serum, when corrected for the small amount of hemoglobin usually present, was equal to the inorganic iron of the serum of both fasting dogs and those given inorganic iron salts. The amount of iron in the serum of dogs in the postabsorptive state varied between 100 and 200 micrograms of Fe per 100 cc. Constant values were observed during fasting. Following the oral administration of ferrous chloride, in doses equivalent to 2.5 to 5.0 mg. of Fe per kilo of body weight, the non-hemoglobin iron of the serum increased. In 1 hour the rise was definite and in 3 to 4 hours peak values of 300 to 550 micrograms per 100 cc. were observed. The concentration of iron then slowly decreased and in 12 to 24 hours, depending on conditions, returned to the fasting level. The oral administration of ferrum reductum also resulted in a rise and a fall of the serum iron.

From the results obtained it is concluded that inorganic iron is absorbed from the alimentary tract into the blood stream where it circulates as inorganic iron in the plasma.

**ON THE ORIGIN OF CREATINE. IV**

BY RICHARD J. BLOCK AND ERWIN BRAND

(From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York)

A relationship of glycine to creatine formation was shown by the *in vivo* experiments of Brand, Harris, and their collaborators.<sup>1</sup>

\* Aided by Grant 209 from the Committee on Therapeutic Research, American Medical Association.

<sup>1</sup> Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I., *Am. J. Physiol.*, **90**, 296 (1929). Harris, M. M., and Brand, E., *J. Am. Med. Assn.*, **101**, 1047 (1933). Brand, E., and Harris, M. M., *Science*, **77**, 589 (1933).

On the other hand, the reports of Abderhalden and Buadze<sup>2</sup> indicated that the incubation of histidine, hydantoin, purines, pyrimidines, and nucleic acid with hashed tissue (liver, kidney, brain, muscle, etc.) results in an augmentation of the total creatinine as determined by the Folin method.

The latter experiments were reinvestigated. Muscle, kidney, liver, and brain were obtained from freshly killed rats and guinea pigs. The hashed tissues alone or in combination with each other were incubated for 24 or 48 hours at 37° with the following compounds and various mixtures of them: arginine, ornithine, glycine, histidine, adenine, nucleic acid, hydantoic acid, hydantoin, methylhydantoic acid, methylhydantoin, and guanidoacetic acid. Experiments, controls, and blanks were carried out in triplicate. The creatinine was determined photometrically<sup>3</sup> on the protein-free filtrates prepared according to Rose.<sup>4</sup>

Contrary to the findings of Abderhalden and Buadze,<sup>2</sup> no increase in the total creatinine over the controls or blanks was observed in any of these experiments. In several cases, notably with liver, incubation at 37° resulted in a decrease in the amount of total creatinine.

### CHOLESTEROL IN MUSCLE

BY W. R. BLOOR AND RUTH H. SNIDER

(From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

Muscles may be grouped according to their cholesterol content. Skeletal muscle, such as that of the front and hind limbs, has the lowest cholesterol content of all muscles; in fact, of all tissues, not excepting the blood. It averages about 0.06 per cent of the moist weight or 0.25 per cent of the dry weight and does not vary much for the same muscle with species or use, while the phospholipid content of the muscle varies with use. The phospholipid to cholesterol ratio is therefore generally higher in the trained muscle

<sup>2</sup> Abderhalden, E., and Buadze, S., *Z. ges. exp. Med.*, **65**, 1 (1929); **66**, 635 (1929).

<sup>3</sup> Kassell, B., *J. Biol. Chem.*, **100**, lviii (1933). Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935), cf. foot-note 3.

<sup>4</sup> Rose, W. C., Helmer, O. M., and Chanutin, A., *J. Biol. Chem.*, **75**, 544 (1927).

than in the untrained one. Those muscles which are more directly concerned with the life processes of the animal, such as those of the jaw or diaphragm, or its equivalent, and the heart, have a considerably higher cholesterol content than the skeletal muscles. Cholesterol of the jaw and diaphragm muscle averages about 0.35 per cent of the dry weight; the heart about 0.65 per cent. Involuntary muscles, such as those of the stomach in mammals or the gizzard in birds, are characterized by a relatively high cholesterol, averaging 0.60 per cent, together with a low phospholipid and a consequently low phospholipid to cholesterol value.

### SERUM PHOSPHATASE, BILIRUBIN, AND CHOLESTEROL IN EXPERIMENTAL JAUNDICE

By AARON BODANSKY AND HENRY L. JAFFE

(From the Laboratory Division, Hospital for Joint Diseases, New York)

Continuing our investigation of hepatogenic, as distinguished from osteogenic or hematogenic increase of serum phosphatase, we ligated the common bile duct in young dogs. Their diet contained commercial biscuit, bread, and milk; they refused meat and liver after the first 2 weeks. The dogs lost weight; moderate anemia developed. The values reported were among those followed in two dogs.

*First Phase, Lasting About 1 Month*—Serum phosphatase reached 150 and 240 units per 100 cc., respectively (80 times the initial values); cholesterol 360 and 470 mg.; and bilirubin 10 mg.

*Second Phase, Lasting About 2 Months*—Phosphatase decreased to 30 and 140 units per 100 cc., respectively; cholesterol to 90 and 190 mg.; and bilirubin to 0.2 and 2.5 mg. The aversion for meat and liver diminished somewhat; after eating some liver one dog developed ascites and edema, which disappeared again on a bread and milk diet.

The beginning of a *third, terminal phase* was indicated by great increases of phosphatase, bilirubin, and cholesterol.

The experiment was terminated in one dog before this ultimately fatal phase advanced to changes reported elsewhere. In this dog we found a small duodenal ulcer and definitely but not strongly jaundiced mucous surfaces, subcutaneous tissues, and abdominal fasciæ. The liver was small, and appeared granular on cut surfaces; cirrhotic changes and some degeneration, but no necrosis, appeared in stained frozen sections.

**OBSERVATIONS ON THE ORIGIN OF CREATINE**

BY MEYER BODANSKY

*(From the Department of Pathological Chemistry, School of Medicine, University of Texas, and the Laboratories of the John Sealy Hospital, Galveston)*

Of a number of alleged precursors of creatine, glycocyamine (Hoffmann-La Roche) was the only compound which produced a creatinuria in any way comparable to that obtained when creatine itself was fed. The albino rat served as the experimental animal and the various substances were administered by stomach tube.

Glycine increased the creatine output, but it required from 10 to 20 times the glycocyamine or creatine dose to produce a significant effect. A smaller and even more variable effect was obtained with alanine, while the results with histidine and glutamic acid were usually negative, except for a slight increase observed occasionally.

Contrary to certain reports in the literature, arginine, aspartic acid, cystine, and tyrosine produced no effect on the creatine excretion. The results with choline, betaine, sarcosine, guanidine, guanidine carbonate, methylguanidine, and creatone (oxalylmethylguanidine) were likewise negative. The creatinine output remained remarkably constant in all the experiments.

The creatinuria resulting from glycine is not offered as proof of the origin of creatine from this amino acid.

**PROPERTIES OF HORMONES IN THE FEMALE URINE**

BY D. E. BOWMAN, J. P. VISSCHER, AND JAMES W. MULL

*(From the Department of Biology and the Laboratory of the Maternity Hospital, Western Reserve University, Cleveland)*

Commercial antuitrin, from the pituitary gland, reduces oxidation-reduction dyes in the upper scale, particularly *o*-chlorophenol indophenol in neutral solution. Using this property, exhibited by the pure antuitrin preparations of Dr. Wallen-Lawrence, as a test, we have studied the presence of antuitrin in the female urine as a possible test for pregnancy. By methods of extraction and concentration suitable for the purpose, over 100 specimens from pregnant, non-pregnant, and doubtful cases have been examined, the latter being checked against the Aschheim-Zondek method.

Detectable quantities of antuitrin were found in urine in 90 per cent



or more of the pregnant cases, none in the normal non-pregnant, but it was present in a few of the doubtful, though non-pregnant, cases. These caused mature follicles and enlargement of the uterus in the Aschheim-Zondek test animals, but no ruptured follicles or corpus luteum. An antuitrin test was also obtained in a majority of urines containing pathological amounts of protein. This may be an error due to the interference of protein, in non-pregnant specimens, or may be merely the result of kidney damage.

Injection, either of commercial or our extracted antuitrin, into a rat produces mature unruptured follicles and enlarged uterus, but no corpus luteum. Antuitrin-S, on the other hand, gives a typical Aschheim-Zondek reaction and its presence in urine is probably an indication of pregnancy. It will reduce oxidation-reduction dyes, under suitable conditions, but must be separated from antuitrin before the test can be applied.

#### **THE RELATION OF AMMONIA SECRETION TO THE ACID-BASE BALANCE OF THE URINE IN DIFFERENT TYPES OF NEPHRITIS**

By A. P. BRIGGS

*(From the Department of Internal Medicine, St. Louis University School of Medicine, St. Louis)*

A study has been made of the relation between the secretion of ammonia and the excess of acid over fixed base, in the urine of normal individuals and patients with different types of nephritis. Interest centers chiefly on the excessive secretion of ammonia which accompanies the nephrotic syndrome, its possible relation to functional activity of the kidney, and to the problem of edema.

#### **THE RELATION OF ADENINE NUCLEOTIDE TO HEMOGLOBIN, HEMATOCRIT, AND RED CELL COUNT IN HUMAN BLOOD**

By MARY V. BUELL

*(From the Chemical Division, Department of Medicine, the Johns Hopkins Hospital and the Johns Hopkins University, Baltimore)*

A study has been made by the Buell method of the adenine nucleotide content of the blood of 100 anemic patients, 100 normal individuals, and a few suffering from polycythemia. Morphologically and etiologically the anemias comprised an extraordinary variety, of varying degrees of severity, in various stages of treat-

ment. Those cases were excluded which were known to be associated with leucemia, carcinoma, nephritis, diabetes, and liver disease; otherwise no selection of data has been made. The extreme limits of hemoglobin concentration observed were 2.5 and 25 gm. per cent.

The nucleotide has been correlated with (1) red cell count, (2) hematocrit, and (3) hemoglobin. No matter what the type, or cause or severity of the condition, the relation of the nucleotide to each of these three factors was unmistakable and remarkably constant over a wide range. The correlation coefficient of nucleotide with red cell count was  $+0.723$ , with hematocrit  $+0.888$ , and with hemoglobin  $+0.898$ . Obviously the correlation of nucleotide with red cell count is secondary to its correlation with hematocrit or hemoglobin.

Whether the primary association is between nucleotide and hemoglobin or between nucleotide and hematocrit cannot be concluded dogmatically. A partial correlation coefficient between nucleotide and hemoglobin, in which the hematocrit was held constant, was found to be  $+0.40$ . This observation, based on 204 cases, lends support to the theory of a possible association between hemoglobin and nucleotide.

### THE METABOLISM OF GLYCINE AND ALANINE

BY JOSEPH S. BUTTS AND MAX S. DUNN

*(From the Department of Biochemistry, University of Southern California School of Medicine, and the Department of Chemistry, University of California at Los Angeles, Los Angeles)*

This is the first of a series of experiments in which the glycogenic action and the ketolytic or ketogenic ability of the various amino acids prepared by one of us (M.S.D.) are investigated. When glycine or *DL*-alanine was fed in amounts corresponding with the carbon atoms to fasting male rats, no rise in glycogen content over the control level was observed in the first case, while a definite rise in liver glycogen occurred 8 hours after alanine.

The administration of glycine to fasting rats in which a ketonuria was induced by the administration of sodium acetoacetate according to the procedure of Butts and Deuel,<sup>5</sup> was much less

<sup>5</sup> Butts, J. S., and Deuel, H. J., Jr., *J. Biol. Chem.*, **100**, 415 (1933).

effective than glucose in bringing about a decrease in the excretion of acetone bodies. On the other hand *dl*-alanine showed a greater ketolytic activity than glycine, but somewhat less than glucose. Studies are in progress to compare the behavior of the *d* and *l* isomers of alanine.

#### THE ABSORPTION OF GLUCOSE AND GALACTOSE FROM THE INTESTINE OF THE DOG

By F. A. CAJORI AND WALTER G. KARR

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

The rates of absorption of glucose and galactose from Thiry loops of the jejunum in dogs have been studied. When solutions containing both these sugars were inserted into the loop, glucose was absorbed more rapidly than galactose, the total carbohydrate absorption being about the same as when the sugars were given separately. However, no difference was found in the rate of absorption of glucose and galactose when they were administered separately.

There was no indication that in the presence of phosphates, the rate of glucose, galactose, or fructose absorption was increased. These results, which are contrary to those reported by Magee and Reid,<sup>6</sup> are of interest in view of the hypothesis that the mechanism of sugar absorption involves phosphorus compounds. The rate of sugar absorption was not changed in the presence of sulfate or chloride.

$\frac{1}{2}$  to 1 hour following the administration of isotonic solutions, the osmolar concentration of the loop contents remained close to the accepted value for dog blood. Water was not absorbed as rapidly as sugar, the absorbed sugar being replaced by chloride. When the solutions introduced into the intestinal loop had an osmotic pressure twice that of blood, an hour's time was hardly sufficient for the attainment of osmotic equilibrium. Analysis showed, however, that chloride had entered the hypertonic solution.

<sup>6</sup> Magee, H. E., and Reid, E., *J. Physiol.*, **73**, 163 (1932).

**SOME ANALYSES OF SAMPLES OF BENCE-JONES PROTEIN**

By HERBERT O. CALVERY AND RICHARD H. FREYBERG

*(From the Departments of Physiological Chemistry and Internal Medicine, Medical School, University of Michigan, Ann Arbor)*

Duplicate analyses were made of different samples of Bence-Jones protein from one individual by some of the standard methods for protein analysis and the following average percentage values have been found: total N 18.1, amino N (per cent of total N) 78.7, P none, S 1.0, amide N 5.6, humin N none, melanin N none, tyrosine 6.8, tryptophane 2.5, cystine 3.0, arginine 5.1, histidine 1.1, lysine 6.8. The nitrogen values are calculated as per cent of the total nitrogen, while all other values are expressed as per cent of the ash- and moisture-free protein. Further analyses will be made as future samples are available in order to determine, if possible, whether all samples are similar in composition.

**CHEMICAL ANALYSIS OF TYPE II PNEUMOCOCCUS SPECIFIC PRECIPITATE**

By HERBERT O. CALVERY, MICHAEL HEIDELBERGER, AND FORREST E. KENDALL

*(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor, and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)*

Two samples of Type II pneumococcus specific precipitate were mixed, ground to a powder, and passed through an 80 mesh sieve. The following analyses were then made (the values are expressed as per cent of the ash- and moisture-free protein): total N 16.0, amino N after acid hydrolysis (as per cent of total N) 75.1, P none, S 1.2, amide N 3.6, humin N 0.12, melanin N 0.50, tyrosine 5.5, tryptophane 2.0, cystine 3.1, arginine 5.5, histidine 1.1, lysine 4.8. The ash content was only 0.17 per cent and the moisture averaged 6.1 per cent. The nitrogen figures are expressed in terms of total nitrogen. Other specific precipitates are being analyzed.

**SOME ANALYSES OF CRYSTALLINE PEPSIN**

By HERBERT O. CALVERY, R. M. HERRIOTT, AND  
J. H. NORTROP

*(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor, and The Rockefeller Institute for Medical Research, Princeton)*

Crystalline pepsin was prepared by the usual method, dialyzed free of sulfates at 10°, and a part of it immediately heat-coagulated at pH 4. A fraction (20 per cent) remained in solution, the significance of which we wished to determine. Consequently analyses have been made of some of the original uncoagulated dialyzed fraction, the heat-coagulated fraction, and the soluble residue from the coagulated material. Analyses for arginine, histidine, lysine, tyrosine, tryptophane, and cystine have been made and some striking differences have been observed, particularly in the tyrosine and cystine contents of these fractions.

**PEPTIC HYDROLYSIS OF EGG ALBUMIN**

By HERBERT O. CALVERY AND ELLEN D. SCHOCK

*(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)*

In a previous investigation of the action of pepsin and other enzymes on crystalline egg albumin it was postulated that the old theory that long chain peptides were the major products of the action of pepsin on proteins is probably incorrect. During the past 2 years further evidence has been found to support this postulate. Fractionations of peptic hydrolysis products have been made in various ways and both chemical and enzymatic studies were carried out on the fractions. As a result another fact has been established which has been reported by other investigators but has not been strongly enough emphasized, and that is that free amino acids are liberated during peptic digestion of proteins. It is believed that this is not due to the acid which is necessary for peptic action.

One of the methods used was dialysis, whereby three fractions were obtained. Each of these fractions, as well as the original solution, has been analyzed for total N, amino N, arginine, histidine, lysine, tyrosine, tryptophane, and cystine and some differences have been noted.

**THE RATE OF CHANGE OF ALKALI RESERVE AFTER INGESTION  
OF SALTS OF ORGANIC COMPOUNDS**

BY JANE CAPE

*(From the Department of Physiological Chemistry, Medical School,  
University of Wisconsin, Madison)*

A previous paper from this laboratory reported studies showing that the influence of ingesting a 6 gm. dose of sodium citrate or its equivalent of sodium bicarbonate upon the acid-base condition of the blood is neither prolonged nor marked.

The investigation has been continued in order to determine what effects, if any, the ingestion of larger amounts of sodium citrate exerted on the acid-base balance. Preliminary observations were made on a few normal subjects under basal conditions to determine the effect of ingesting 12 gm. of sodium citrate. Another series on six subjects was made to ascertain effects of a 20 gm. dosage.

The levels for all three values, pH, CO<sub>2</sub>, and total base, in these observations where 12 gm. were used showed no significant variations.

The maximum response varied from 90 minutes to 2 hours with different individuals on the 20 gm. dosage. The CO<sub>2</sub> content increased on the average of 9.3 volumes per cent; for pH, 0.07 to 0.09; whereas the total base remained more constant or within the range of normal variations, as determined by the authors in a previous paper.

**THE THYROXINE AND IODINE CONTENT OF NORMAL AND  
PATHOLOGICAL THYROGLOBULIN**

BY J. W. CAVETT, CARL O. RICE, AND J. F. McCLENDON

*(From the Laboratory of Physiology and the Department of Medicine,  
University of Minnesota, Minneapolis)*

Thyroid glands were classified clinically and histologically, and thyroglobulin was isolated and purified by the method of Cavett and Seljeskog. Van Slyke nitrogen distribution with direct histidine determination does not show a difference between thyroglobulins from normal and pathological glands but *the latter are lower in thyroxine*. The thyroglobulin obtained at autopsy from groups of glands (weighing 15 to 40 gm. each) from normal cases

in which no iodine medication had been used averaged 0.33 per cent thyroxine. The pathological glands were obtained at operation. Colloid goiters without iodine medication contained about 0.015 per cent thyroxine, whereas after 4.4 gm. of iodine (as Lugol's solution) were given preoperatively, only 0.082 per cent thyroxine was found in the thyroglobulin. The thyroglobulin from adenomatous goiters, whether iodine medication was used or not, contained *less thyroxine than that of normal thyroglobulin*. All exophthalmic cases received iodine preoperatively but the *thyroxine content of the thyroglobulin was lower than normal*. When classified according to thyroxine content of thyroglobulin, the colloid, adenomatous, and exophthalmic goiters show no distinctive character, the only difference being whether they were from patients given iodine medication or not, despite the great difference in total iodine content as shown in the following:

Total iodine of the thyroglobulin of "normal" glands averaged 0.39 per cent; of colloid goiters without medication, 0.045 per cent; with 4.4 gm. of iodine medication, 0.25 per cent; from adenomas without medication, 0.07 per cent; with medication, 0.2 to 0.4 per cent; exophthalmic goiters, 0.2 to 0.57 per cent; whereas in "normal" thyroids half, in goiters less than one-third, of the thyroglobulin-iodine is thyroxine-iodine and iodine medication of goiters causes formation of diiodotyrosine or some other non-thyroxine thyroglobulin constituent.

#### RENAL FUNCTION STUDIES IN PARTIALLY NEPHRECTOMIZED RATS

By ALFRED CHANUTIN AND STEPHAN LUDEWIG

(From the Laboratory of Physiological Chemistry, University of Virginia, University)

The results obtained in a modified urea clearance test and a concentration test were compared in partially nephrectomized rats fed diets containing 10, 20, 40, and 80 per cent whole dried meat as the source of protein. The "urea test" was found to be a good indicator of the degree of kidney damage. The urinary specific gravity served as a good qualitative measure of kidney function. The blood urea concentration increased markedly in these animals only after the "urea ratio" was lowered. There was a definite tendency toward higher volumes of urine during the

concentration test with the low "urea ratios." There was no relationship between the degree of proteinuria and the kidney function. The time required to reduce the "urea ratio" was affected by the amount of meat in the diet. Those animals receiving diets containing 10 per cent meat had the best kidney function as judged by the "urea ratio" and the concentration test.

**CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE VARIOUS FRACTIONS OF THE BACILLUS CALMETTE-GUÉRIN (BCG)\***

BY ERWIN CHARGAFF AND WERNER SCHAEFER

*(From the Pasteur Institute, Paris, France)*

In continuation of previous work on the chemistry of BCG, the chemical and serological properties of the various fractions were studied. Among the lipid fractions (fat, phosphatide, wax) the phosphatide alone showed a very distinct immunological activity. This substance is in many respects very similar to the phosphatides prepared by R. J. Anderson and his collaborators from pathogenic acid-fast bacteria. It is an ester of a phosphorylated mannose-containing polysaccharide with palmitic acid and liquid saturated fatty acids of high molecular weight.

This phosphatide, when injected into animals, acted as a true antigen and produced antibodies. It also reacted with anti-BCG horse serum, as shown by complement fixation tests. In this way 0.3 microgram of the phosphatide, which is not type-specific, could still be demonstrated. It was shown by chemical fractionation experiments that this activity was not due to impurities adhering to the phosphatide, but that the phosphatide itself was the antigen.

Polysaccharide preparations from BCG were likewise examined. The polysaccharides extracted from the defatted bacteria by means of phosphate buffers or dilute acetic acid were found to act as haptenes, giving precipitates with anti-BCG horse sera up to a dilution of 1:1,000,000. Antibodies were not produced. Another polysaccharide which accompanies the lipids extracted by alcohol-ether (corresponding to Anderson's Fraction A-8) was devoid of any serological activity.

\* Aided by a grant to one of the authors (E. C.) from the Rockefeller Foundation.



**A METHOD FOR THE DETECTION AND DETERMINATION OF  
CARBON MONOXIDE IN AIR**

BY A. A. CHRISTMAN AND WALTER D. BLOCK

*(From the Department of Physiological Chemistry, Medical School,  
University of Michigan, Ann Arbor)*

Concentrations of carbon monoxide in air ranging from 0.02 to 0.06 per cent may be determined with an accuracy of  $\pm 5$  per cent with a 500 cc. sample of air. Higher concentrations of carbon monoxide may be determined with the same accuracy by the use of smaller samples of air. 1 part of carbon monoxide in 25,000 parts of air (0.004 per cent) may be qualitatively detected. The determination is based on the reduction of palladium chloride to metallic palladium by carbon monoxide and the determination of the excess palladium chloride by a colorimetric procedure.

**CONDITIONS INFLUENCING THE STORAGE OF VITAMIN A IN  
THE RAT**

BY S. W. CLAUSEN AND A. B. McCOORD

*(From the Department of Pediatrics, The University of Rochester School of  
Medicine and Dentistry, Rochester, New York)*

Among the various factors which might influence the storage of vitamin A in the rat, chloroform poisoning has been studied. When the test animals are first severely poisoned with chloroform and then given a massive dose of vitamin A in the form of haliver oil 48 hours later, the amount of vitamin A recovered from their tissues is sometimes less than that of their controls, apparently owing to faulty absorption through the injured intestinal walls. On the other hand, if the vitamin A is administered first, and the rats poisoned with chloroform 24 hours later, no destruction in the vitamin occurs.

In agreement with the recent work of Baumann, Riising, and Steenbock, it is found that the more extreme the vitamin A depletion of rats, the less the amount of a large dose of vitamin A recovered from their livers. Extending the work of these authors, we have found that the loss is also reflected in the lungs of the rats, but the quantities of vitamin A recovered in the adrenals are far above those in less severely depleted rats. This latter fact seems to indicate that the compound may be absorbed and may not undergo excessive destruction in the digestive tract.

Preliminary experiments indicate that vitamin A is destroyed when rats are subjected to artificial fevers, the initial loss taking place in the lungs and adrenals.

#### **ACTION OF CERTAIN OXIDATIVE STIMULANTS AND DEPRESSANTS ON RESPIRATION AND CELL DIVISION**

By G. H. A. CLOWES AND M. E. KRAHL

*(From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, and the Marine Biological Laboratory, Woods Hole)*

4,6-Dinitro-*o*-cresol at a concentration of  $5 \times 10^{-6}$  M exerts a maximum stimulation of respiration in sea urchin eggs amounting to some 400 per cent in fertilized and 600 per cent in unfertilized eggs. At this concentration the division of fertilized eggs is markedly retarded and at higher concentrations suppressed without, however, any injury to the eggs which, even after several hours exposure to higher concentrations of the reagent, continue division in a normal manner when returned to sea water. Cyanides, which both lower respiration and block division, act antagonistically to 4,6-dinitro-*o*-cresol so far as respiration is concerned but additively so far as the block to division is concerned. When egg development is blocked by 4,6-dinitro-*o*-cresol, the great majority of eggs is found on examination to have reached the early prophase of division, but they differ from normal eggs in the early prophase in that the nucleus is spherical in form and the membrane does not show any signs of commencing disintegration, in spite of the fact that the chromosomes are easily recognizable and fully as large and as clearly stained as are the chromosomes in normal prophase. A similar tendency to a block in the early prophase is observed with concentrations of cyanide high enough to inhibit development but not to cause destruction of the egg.

#### **THE SOLVENT ACTION OF NEUTRAL SALTS UPON PEPTIDES IN SOLUTIONS OF LOW DIELECTRIC CONSTANT**

By EDWIN J. COHN, THOMAS L. McMEEKIN, AND  
JESSE P. GREENSTEIN

*(From the Department of Physical Chemistry, Harvard Medical School, Boston)*

The principle of the ionic strength, first deduced from the study of globulins, although adequate for the description of interionic

forces, in dilute aqueous solution, does not generally suffice for the description of the solvent action of neutral salts upon amino acids and peptides in solvents of high dielectric constant. Specific salt effects are manifested at far lower ionic strengths in the interaction of ions and zwitter ions than of ions with each other.

The principle of the ionic strength is adequate to describe the behavior of peptides in such alcohol-water mixtures that the ratio  $(D/D^0 \log N/N')/((D^0/D) \mu)$  is independent of the dielectric constant. The solvent action of salts on the peptides of glycine and upon lysylglutamic acid has been studied under these conditions. The ratios calculated quantitatively measure the electrostatic forces due to these peptides. They are greater the greater the electric moment of the zwitter ion, and the greater the ionic strength of the solution, as demanded by the theories of Scatchard and Kirkwood.

#### IODINE BALANCE STUDIES ON THE WHITE RAT

BY VERSA V. COLE AND GEORGE M. CURTIS

*(From the Department of Medical and Surgical Research, the Ohio State University, Columbus)*

Iodine balance studies are reported on normal rats, on rats receiving thyrotropic hormone, and on thyroidectomized rats. Adult male rats were used throughout. 4 day metabolism periods were used. Three to six consecutive periods were run on each group of rats. Thyrotropic hormone was prepared from beef pituitaries by Loesser's method. The material was given intraperitoneally daily to one group of rats and another group of rats was totally thyroidectomized. Carbon dioxide output was followed on all three groups of rats. At the end of each experiment, the animals were sacrificed. Thyroids from those not previously thyroidectomized were removed. One-half of each thyroid was used for histological sections, and one-half for iodine analysis. The remainder of the animal was dried and ground and a sample taken for iodine analysis. Hyperplasia and low iodine content of the thyroid were taken as evidence of hyperthyroidism. A comparison of the iodine balance and a discussion of the relationship of the thyroid to iodine metabolism are presented.

**FACTORS INFLUENCING THE CREATINE CONTENT OF THE MUSCLES**

BY RALPH C. CORLEY, ALICE W. KRAMER, AND PAUL A. WOLF

*(From the Division of Biochemistry, Department of Chemistry, Purdue University, West Lafayette)*

Others have found it quite difficult to affect the creatine content of the muscles of healthy animals by dietary means, other than by the administration of creatine itself. The influence of other substances is controverted. It would appear that a profitable method of attack on the origin of creatine would be to study the influence of the possible precursors or relatives of creatine in animals treated with substances found to alter consistently the creatine of the tissues.

We have therefore studied the effect on the creatine of the muscles of white rats of substances which have been reported to influence the creatine of tissues or to influence the excretion of creatine, or which by induction or analogy might be expected to be of influence. We have rechecked the effect of fasting and of diets of varying protein content. We have studied the influence of the administration, respectively, of parathormone, adrenalin, ephedrine, phosphorus, sodium fluoride, iodoacetic acid, phlo-rhizin, thyroid tissue, dinitrophenol, and guanidine.

Creatine, glycine, glutamic acid, alanine, and arginine, respectively, have been fed to rats treated with phosphorus, parathormone, or ephedrine. A number of interesting correlations have been found.

**THE METABOLISM OF BROMINE AND OF BROMINATED FATTY ACIDS IN THE ANIMAL BODY**

BY RALPH C. CORLEY, JOHN T. TRIPP, AND EDITH R. NEWTON

*(From the Division of Biochemistry, Department of Chemistry, Purdue University, West Lafayette)*

We have been studying the metabolism of brominated fatty acids to learn more of the mechanism of the oxidation of fatty acids in the animal body. Tetrabromostearic acid, in doses of as much as 100 to 150 gm., has been ingested by healthy human subjects without deleterious symptoms. The compound is broken down in the body to yield in the urine a product or products con-

taining organically bound bromine. Isolation preliminary to identification is in progress.

As the carcass of a rat contains considerable amounts of organically bound bromine after the ingestion of tetrabromostearic acid, it has been of interest to determine the bromine content of various tissues after the administration of brominated fatty acids. White rats have ingested, respectively, tetrabromostearic acid, dibromobehenic acid, dibromostearic acid, and potassium bromide.

Nine tissues, including the thyroid, hypophysis, and lungs, have been analyzed for bromine by a recently reported accurate electro-metric method for the determination of bromides in the presence of chlorides. All the tissues contained distinctly more bromine than the controls. A number of interesting relationships in the distribution of bromine appear.

#### THE INFLUENCE OF *l*- AND *dl*-TRYPTOPHANE AND KYNURENIC ACID ADMINISTRATION ON BILE VOLUME AND COMPOSITION

BY JOHN T. CORRELL, CLARENCE P. BERG, AND  
DONALD W. COWAN

(*From the Biochemical and Physiological Laboratories, State University of Iowa, Iowa City*)

According to Whipple and Smith, tryptophane given to bile fistula dogs acts as a cholagogue; also, when fed with certain proteins, it increases bile salt production. Appreciable quantities of kynurenic acid appear in the bile, as well as in the urine, after tryptophane administration (Kotake and Ichihara). We have undertaken to determine whether kynurenic acid production or excretion might be responsible, at least in part, for the cholagogue effect of tryptophane administration, and also, to study the influence of optical configuration of tryptophane on bile volume and on bile salt output.

The observations of the workers mentioned have been confirmed. Apparently, *dl*-tryptophane, which produces less kynurenic acid than does an equal amount of *l*-tryptophane, is also less effective as a cholagogue. Further studies on the effect of *dl*-tryptophane and of kynurenic acid on bile production are in progress.

## AMINO ACID CONTENT OF STAPLE FOODS

BY FRANK A. CSONKA

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils,  
United States Department of Agriculture, Washington)

There are recorded in the literature a large number of proteins which have been isolated from most of the commonly used food materials. In the study of these proteins consideration has heretofore been given chiefly to their chemical and physical characterization, and their amino acid composition. There is but little known regarding the relative proportions of the different proteins present in the various materials studied; consequently there is no basis upon which the amount of any given amino acid in the material as a whole can be estimated. The aim of the work described in this paper was to develop a suitable method to determine quantitatively the total cystine, tryptophane, histidine, arginine, lysine, and tyrosine content in some of the staple foods, and to express the values in terms of the total nitrogen in the material studied. Such data will find practical application in adjusting and correcting certain amino acid deficiencies in foods by proper supplementation.

Wheat flour was successively extracted with 1 per cent sodium chloride solution, 65 to 70 per cent alcohol, and 20 per cent hydrochloric acid at refrigerator temperature. Starch was eliminated from the acid extract by addition of alcohol. In this manner all the nitrogen in the flour was removed, and the amino acids quantitatively determined. Work on other cereals and seeds is in progress.

## CARBAMINO COMPOUNDS AND CARBON DIOXIDE TRANSPORT

BY CORNELIUS A. DALY AND D. B. DILL

(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

Ferguson and Roughton<sup>7</sup> find that carbamino combination of CO<sub>2</sub> and hemoglobin is favored by (a) diminishing oxygen, (b) decreasing temperature, and (c) increasing alkalinity. Evidently hemoglobin can combine with more CO<sub>2</sub> than with other acids or else carbamino formation is at the expense of HCO<sub>3</sub><sup>-</sup>. Experi-

<sup>7</sup> Ferguson, J. K. W., and Roughton, F. J. W., *J. Physiol.*, **83**, 87 (1934).

ments of Van Slyke and Hawkins<sup>8</sup> and of Stadie and O'Brien<sup>9</sup> render the first alternative improbable. We have given it a further test by titrating Hb and HbO<sub>2</sub> solutions, freed of CO<sub>2</sub> by evacuation, with CO<sub>2</sub> and HCl at 0.8°, 20°, and 37.5°, using the glass electrode for all pH determinations. The curves from pH 8.3 to 6.7 are identical for the two acids, the slopes are independent of temperature, and the effect of oxygenation is the same for the three temperatures, as well as for the two acids. It is clear that if carbamino compounds are present when equilibrium between CO<sub>2</sub> and Hb or HbO<sub>2</sub> is reached, an equivalent amount of HCO<sub>3</sub><sup>-</sup> is sacrificed.

Carbamino compounds appear to serve no useful physiological purpose unless their rapid transient formation in the capillaries occurs. Even here they are not essential. For example, little carbamino CO<sub>2</sub> can exist at the end of exhausting exercise, because the pH<sub>c</sub> in venous blood may be 6.7. Yet here CO<sub>2</sub> transport may be 15 cc. per 100 cc. of blood or 3 times the resting rate.

#### BLOOD SUGAR IN RATS IN WHICH CATARACT WAS PRODUCED BY A VITAMIN G-DEFICIENT DIET AND BY A LACTOSE- CONTAINING DIET

By PAUL L. DAY

(From the Department of Physiological Chemistry, School of Medicine,  
University of Arkansas, Little Rock)

Young rats given an adequate diet containing 60 per cent lactose developed cataract, as reported by Mitchell and Dodge. Rats receiving similar diets containing glucose, sucrose, or starch in place of the lactose did not exhibit lenticular changes. At intervals during the experiment blood was obtained from the tail and sugar was determined by the Folin micromethod. The rats receiving lactose showed higher sugar levels than litter mates receiving the other carbohydrates. This hyperglycemia tended to become less marked as the rats reached maturity. Since some authorities believe that hyperglycemia is an etiological factor in the development of diabetic cataract, it is possible that the mechanisms for the formation of this lactose cataract and diabetic cataract are the same.

<sup>8</sup> Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, **87**, 265 (1930).

<sup>9</sup> Stadie, W. C., and O'Brien, H., *Biochem. Z.*, **237**, 290 (1931).

Rats rendered cataractous by a vitamin G-deficient diet did not have elevated blood sugar levels. The conclusion seems warranted, therefore, that abnormal carbohydrate metabolism could hardly be an etiological factor in the formation of the cataract resulting from vitamin G deprivation.

#### ERRORS IN CALCULATED pH IN HEMOGLOBIN SOLUTIONS WITH LITTLE AVAILABLE BASE

By D. B. DILL, W. H. FORBES, AND L. J. HENDERSON

(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

Among the studies of the possible combination of  $\text{CO}_2$  and hemoglobin made by various investigators, there is none which deals fully with solutions containing little available base. Margaria and Green<sup>10</sup> report two experiments in which the base was low but their  $\text{CO}_2$  pressures were not below 9 mm.

We have performed a series of experiments upon Hb,  $\text{HbO}_2$ , and MetHb in solutions containing very small quantities of base and with  $\text{CO}_2$  pressures as low as 0.2 mm. There is a marked difference under these conditions between the pH as calculated by the Henderson-Hasselbalch equation and as measured by the glass electrode—a difference amounting to 1.0 unit in extreme cases, the calculated pH being the higher. This was true for all three solutions.

There are three alternative explanations: (1)  $\log [\text{H}\text{HCO}_3]$  is too large or (2)  $\log [\text{H}_2\text{CO}_3]$  is too small or (3) the value of  $\text{pK}'_1$  decreases more and more rapidly as  $\text{pCO}_2$  diminishes. The linear relationship between  $\text{pK}'_1$  and pH reported by Margaria and Green did not hold for our solutions below 20 mm. of  $\text{pCO}_2$ . Similar experiments upon glycine solutions gave results which were of the same general nature, though they differed in certain particulars.

#### THE FATE OF HEMOGLOBIN INJECTED INTO THE BLOOD STREAM

By DAVID L. DRABKIN, A. H. WIDERMAN, AND H. LANDOW

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

Approximately 30 cc. samples of hemolyzed washed blood cells (1:4) were injected intravenously into the same dogs from which

<sup>10</sup> Margaria, R., and Green, A. A., *J. Biol. Chem.*, **102**, 611 (1933).



the blood was obtained. The fate of the injected blood pigment was followed during the first 2 to 3 hours by periodic examination of the serum and of the urine obtained by means of catheterization.

By means of spectrophotometry we have been able to determine for the first time quantitatively certain aspects of this phase of pigment metabolism, as well as to recognize the nature of any pigment changes involved. The following points were established: (1) The material which appeared in the urine was unchanged hemoglobin. (2) Upon standing exposed to air, the hemoglobin in the urine was converted rapidly into methemoglobin. (3) The concentration of pigment in the urine was such as to suggest that the excretion was dependent upon its concentration in the blood serum. (4) The amount of hemoglobin excreted in the urine was relatively small. The highest percentage eliminated in the urine was 9.5 of the total quantity injected. One of the animals consistently excreted less than 1 per cent of the pigment. The approximate threshold value for hemoglobin appeared to be 0.1 milli-equivalent per liter of serum. (5) The hemoglobin which appeared in the urine accounted for only one-tenth of the quantity which left the serum during the same period. During the first 2 hours methemoglobin formation in serum was not found.

## STUDIES OF PHENOL ESTIMATION

### I. PRELIMINARY SURVEY

By BEATRICE G. EDWARDS

*(From the Department of Biochemistry and Pharmacology, University of Oklahoma Medical School, Oklahoma City)*

The Folin-Denis, diazotized *p*-nitroaniline and halogen reactions are the only well known phenol tests which can serve as general analytical methods. Most phenol tests are not specific, being given also by quinones, quinhydrones, enolic substances, amines, aldehydes, cyclic alcohols, or carbohydrate derivatives; and the less specific ones by many aromatic compounds.

We have studied the quantitative behavior of many phenols and nitrogenous substances with these reagents. Phenol equivalents have been determined and found to be higher for the Folin-Denis method than the *p*-nitroaniline method. This difference increases for di- and polyphenols. Similar equivalents have been deter-

mined for aldehydes, amines, amino acids, cyclic alcohols, purines, quinhydrone, and quinones by both methods. The *p*-nitroaniline method reacts also with imidazoles, aromatic acids, and sugar derivatives. The lower values for phenols in biological fluids found by Theis and Benedict and by Marenzi with *p*-nitroaniline methods are due to lower phenol equivalents, not to greater specificity.

A direct bromine titration and an indirect bromine number for phenols have also been studied. These estimations are even less specific, being affected by many enolic, unsaturated, and reducing substances. They possess differentiating value, but their usefulness as analytical reagents is limited. Lloyd's reagent, although partially removing certain polyphenols from solution, is well suited for the preparation of analytical filtrates. Other analytical improvements are the addition of gum ghatti to the *p*-nitroaniline reagents, and the replacement of phenol standards by tyrosine solutions, which are more easily prepared and possess greater stability.

#### THE VITAMIN B (B<sub>1</sub>) CONTENT OF ANIMAL TISSUES

By C. A. ELVEHJEM, W. C. SHERMAN, AND AARON ARNOLD

*(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)*

The distribution of vitamin B in animal tissues is of importance not only from the point of view of nutrition, but also because recent studies have indicated that vitamin B is associated with respiration, especially lactic acid oxidation, in certain tissues. Carefully prepared tissues such as brain, spinal cord, liver, lung, pancreas, cardiac and skeletal muscle, kidney from beef, hog, and sheep have been assayed. The potency is based upon the ability of the different preparations to prevent polyneuritis in chicks on an autoclaved natural ration. The activity is compared with that of crystalline vitamin B and standard yeast. Pork muscle, heart muscle, and kidney are fairly rich in vitamin B. Beef muscle, mutton muscle, brain, and lung are very low in this factor. There is no correlation between the vitamin content and rate of respiration of the different types of tissue. The effect of canning upon the destruction of vitamin B has been determined. As high as 80

per cent of the vitamin present may be destroyed under the more drastic methods of commercial canning.

**LIPID AND MINERAL DISTRIBUTION BETWEEN THE RED BLOOD CELLS AND PLASMA IN NORMAL CHILDREN AND IN ANEMIAS OF CHILDHOOD**

BY BETTY NIMS ERICKSON, FRANCES COPE, HELEN R.  
STERNBERGER, PEARL LEE, THOMAS B. COOLEY,  
AND ICIE G. MACY

*(From the Research Laboratory of The Children's Fund of Michigan and the Children's Hospital of Michigan, Detroit)*

Some of the anemias of childhood, namely sickle cell, erythroblastic, hemolytic icterus, and hypochromic, and occasional cases of pernicious and hypochromic anemias of adults have been investigated through detailed hematological, chemical, and physico-chemical studies. The gasometric microlipid methods of Van Slyke and coworkers have been applied in studying the distribution of total lipids, neutral fat, cholesterol, cholesterol esters, and phospholipids in the red blood cells and plasma of anemic and normal individuals.

These observations are accompanied by determination of sodium, potassium, and chloride in the erythrocytes and plasma; and by respiratory, cataphoretic, and fragility studies of the red cells.

The results to date indicate strikingly elevated values of total lipids and phospholipids in the cells of sickle cell and erythroblastic anemias as contrasted with low values in hypochromic and pernicious anemias. No significant differences have been found in the lipid distribution of the plasma of any of the blood dyscrasias observed except pernicious anemia, where the cholesterol and cholesterol esters are very low.

Attempts are being made to relate the chemical composition of the cells and their environment to properties such as resistance to hemolysis, permeability, velocity, respiration, and abnormal hematology with the objective of gaining information about the chemical structure of the red cell.

**THE DETERMINATION OF IRON IN BIOLOGICAL MATERIALS**

BY G. E. FARRAR, JR.

*(From the Thomas Henry Simpson Memorial Institute for Medical Research,  
Ann Arbor)*

The difficulty encountered by many authors in the analysis of iron in biological materials has been experienced in this laboratory. The following methods of estimation have been tested: (1) that proposed by Elvehjem which eliminates the interference of pyrophosphates with the ferric thiocyanate color formation by hydrolyzing the solution with sodium hydroxide; (2) Hanzal's method which removes interfering substances by means of a cupferron precipitation and develops a color with thioglycolic acid; (3) Lintzel's procedure which separates the excess of phosphates by precipitating the iron as ferric phosphate; (4) the method advocated by Stugart which in utilizing small aliquot portions (0.01 mg. of iron) avoids the effect of orthophosphates and causes hydrolysis of the pyrophosphate with hydrochloric acid.

Each of these methods yielded accurate results, provided certain precautions were observed carefully. These methods have been tested on a wide variety of materials: foods, blood, feces, urine. The loss of iron during dry ignition has been studied and eliminated by adding calcium carbonate to maintain an alkaline reaction in the sample. The wet ashing method has been discarded because of unavoidable contamination with iron in the reagents.

The procedure described by Stugart and employed by Elvehjem has been adopted as the preferable method, because it is the simplest adequate way of eliminating the effect of phosphates on the development of the ferric thiocyanate color and because of the very few, easily purified reagents employed.

**CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF SILK PROTEINS**

BY NORBERT H. FELL

*(From the Laboratory of Physiological Chemistry, Medical School,  
University of Michigan, Ann Arbor)*

Silk fibroin, dispersed in lithium bromide and then dialyzed free from salt, functions as a true antigen in immunological reactions. Sera of high titer can be produced in rabbits, and guinea pigs can be sensitized and shocked with fibroin dialysates.

Fibroin is not heat-coagulable, and boiling it in lithium bromide solution does not destroy its immunological properties. When the lithium bromide dispersion is dialyzed in collodion membranes, the resulting solutions frequently gel in a few hours; but fairly stable solutions may be produced by dialyzing against very dilute solutions containing organic anions.

The salt-free fibroin dialysate treated with absolute alcohol yields a compound almost identical chemically with fibroin. The nitrogen and tyrosine values are slightly lower, but the compound will cross-react with anti-fibroin-rabbit serum.

70 minute hydrolysis of fibroin with 70 per cent sulfuric acid at room temperature gives a mixture of fractions, the insoluble portion having a much higher tyrosine content (17 to 19 per cent) than fibroin (12 per cent). This insoluble fraction dispersed in lithium bromide is completely dialyzable, and, as judged by the anaphylactic reaction, it is not antigenic. 30 minute hydrolysis under similar conditions yields an insoluble product with a percentage of tyrosine higher than that of fibroin. This insoluble compound dispersed in lithium bromide will not dialyze; its antigenic properties have not yet been determined, but it does not give a precipitate with anti-fibroin-rabbit serum.

Silk sericin also can be dispersed in lithium bromide, and, after dialysis, it apparently acts as an antigen; but it gives no reaction with anti-fibroin-rabbit serum.

#### THE SOLVENT ACTION OF NEUTRAL SALTS UPON ALBUMINS IN SOLUTIONS OF LOW DIELECTRIC CONSTANT

By RONALD M. FERRY, EDWIN J. COHN, AND ETHEL S. NEWMAN

*(From the Department of Physical Chemistry, Harvard Medical School, Boston)*

The extremely high solubility of albumins presumably depends upon their having electric moments which are great in comparison with their molecular diameters. In media of low dielectric constant, in which they are only slightly soluble, they behave much like globulins, in that their solubility is greatly increased by neutral salts.

The solubility of highly purified egg albumin has been studied as a function of the ionic strength in alcohol-water mixtures at  $-5^{\circ}$ . Under these conditions denaturation of the protein is virtu-

ally eliminated, solubility becomes essentially constant, and activity coefficients may therefore be calculated. Our measurements suggest that the logarithm of the activity coefficient is a function of the ionic strength, as demanded by the theory of Scatchard and Kirkwood, and not of its square root. This observation supports the conception that proteins are zwitter ions in the neighborhood of their isoelectric point.

The estimated electrostatic forces, interpreted on the basis of Kirkwood's theory, give information concerning the distribution of the charged ammonium and carboxyl groups. Electrostatic forces are diminished in dilute aqueous solution and to an even greater extent in concentrated egg albumin solutions, in which the dielectric constant is greater than that of water because of the electric moments due to the same distribution of the charged groups of the protein molecule. The same methods are being employed in the characterization of other proteins.

#### STUDIES ON UROCHROME

By THOMAS A. FLEMING AND A. BRUCE MACALLUM

*(From the Chemical Laboratory, Institute of Public Health, and the Biochemical Department, Faculty of Medicine, University of Western Ontario, London, Canada)*

The urinary concentrates produced in the course of separation of theelin give excellent yields of urochrome which can be obtained from the residues of the first stage and the final step in the process of the separation of the hormone. The urochrome can be separated from tarry residues by dissolving them in alcohol, diluting with an equal volume of water, adding ammonia till slightly alkaline, and precipitating with copper sulfate. When the pigments are in aqueous solution, copper sulfate will precipitate them. The copper urochrome can be decomposed with hydrogen sulfide. Urobilin is not brought down by copper but remains in solution.

An elementary analysis gives C 59, H 7, O 25.9, N 6.6, S 1.5 per cent; which compares with Fischer's<sup>11</sup> analyses, C 52, H 6, O 29, N 11, S 2 per cent.

It is soluble in acids and alkalies, ethyl and butyl alcohol, phenol, acetic acid, and ethyl acetate, slightly soluble in water and acetone, insoluble in ether, benzene, and chloroform.

<sup>11</sup> Fischer, H., and Zerweck, W., *Z. physiol. Chem.*, **137**, 176 (1924).

It is an abiuret compound, which upon hydrolysis yields amino acids and appears to be in the nature of a peptide. It is pyrrole-free, and x-ray analysis indicates an amorphous structure. A faint absorption band exists whose peak is 3200 Å. (water). Cryoscopic measurement with phenol as a solvent gave an apparent molecular weight of 127.

The chemical structure and the rôle of this substance in the oxidation-reduction system of the tissues is under investigation.

#### THE REACTION OF ALDEHYDES AND SUGARS WITH ACETOACETIC ACID

BY THEODORE E. FRIEDEMANN AND ROSALIND KLAAS

*(From the Laboratory of Biological Chemistry, Washington University Medical School, St. Louis, and the Department of Medicine, University of Chicago, Chicago)*

The reaction was studied under two conditions: (1) in non-oxidative solution (Knoevenagel's reaction) and (2) in alkaline  $\text{H}_2\text{O}_2$  (Shaffer's reaction). The former occurs only with aldehydes and in rate and extent in approximately the following order: formaldehyde, glyoxylic acid, glyoxal, methylglyoxal, glyoxal carboxylic acid, glucosone, glycolaldehyde, glyceraldehyde, acetaldehyde, furfuraldehyde, butyraldehyde, aldol, heptaldehyde, benzaldehyde, phenylacetaldehyde, and cinnamic aldehyde. Ketones do not react. The reaction is catalyzed by alkali, but not apparently by nitrogenous bases, as in the case of acetoacetic ester. Formaldehyde combines with only 1 mole of acetoacetate, while glyoxylic acid, glyoxal, and acetaldehyde combine with 2 moles of acetoacetate. The reaction product is unstable and is readily decarboxylated, especially in acid solution. Complete dissociation into aldehyde and acetoacetate can be shown on adding a solution of the immediate condensation compound of glycolaldehyde or glyceraldehyde with acetoacetate either to strong alkali or to alkaline  $\text{H}_2\text{O}_2$ .

The following ketoses and aldoses react with acetoacetate in alkaline  $\text{H}_2\text{O}_2$ : glucose, fructose, mannose, xylose, arabinose, glyceraldehyde, dihydroxyacetone, and glycolaldehyde. Sucrose and substituted sugars, such as acetylmethylcarbinol, rhamnose, and tetramethylglucose, gave negative results. Each ketolytically active sugar brings about the oxidation of 2 moles of acetoacetate.

Although rapidly oxidized, glycol and glyceraldehydes in Fehling's solution have no ketolytic action. These sugars, as in the case of glucose, do not react in  $\text{Na}_2\text{HPO}_4$  and  $\text{H}_2\text{O}_2$  solution; strong alkalinity and  $\text{H}_2\text{O}_2$  are necessary.

These experiments indicate different mechanisms for the non-oxidative and oxidative reactions.

### ISOLATION EXPERIMENTS ON SERUM CREATININE

By OLIVER HENRY GAEBLER

*(From the Department of Laboratories, Henry Ford Hospital, Detroit)*

Most of the apparent creatinine present in cellophane ultrafiltrates of nephritic sera can be precipitated by saturating the ultrafiltrate with picric acid and adding potassium chloride. From 1.8 to 2.7 mg. of apparent creatinine per 100 cc. remained unprecipitated in the case of six ultrafiltrates in which the apparent creatinine values before precipitation were 4.1 to 32.2 mg. per 100 cc. Creatinine added to normal sera before ultrafiltration in amounts of 3 to 11.8 mg. per 100 cc. of serum can also be precipitated from the ultrafiltrates in this way, although in the case of the smaller amounts several days may be required for precipitation.

In the procedure the sera are ultrafiltered through No. 300 cellophane under a high nitrogen pressure. To 10 cc. of ultrafiltrate in a test-tube one adds 250 mg. of pure picric acid. The tube is immersed in hot water and shaken until the picric acid has dissolved. After cooling to room temperature, 0.1 cc. of 10 per cent potassium chloride is added and the solution is shaken at once. If retention is very marked, precipitation of the apparent creatinine may begin at once and go to completion within an hour, while from ultrafiltrates containing about 4 mg. of apparent creatinine per 100 cc. a precipitate of picric acid containing no chromogenic substance separated first, and was removed by centrifugation and decanting. From the decanted liquid a small crop of crystals containing the precipitable portion of the apparent creatinine then separated in the course of 24 hours.



**THE REACTION BETWEEN GOSSYPOL AND CALCIUM AND ITS  
PHYSIOLOGICAL IMPORTANCE**

BY WILLIS D. GALLUP AND RUTH REDER

*(From the Department of Agricultural Chemistry Research, Oklahoma  
Agricultural Experiment Station, Stillwater)*

The first evidence of a reaction between gossypol, the toxic principle of cottonseed, and calcium was obtained in studies of the effect of gossypol on the hydrolysis of fat by lipase. These studies were carried out with an extract of pig pancreas acting on an emulsion of olive oil, at an initial pH of 8.9. Calcium chloride (10 mg.) and albumin were used as activators. Gossypol was added in varying amounts.

In the absence of calcium, hydrolysis was inhibited slightly by gossypol. In the presence of calcium, inhibition by gossypol was marked, and when 6 to 8 mg. of gossypol were dissolved in the oil previous to emulsification, no hydrolysis took place. In similar studies carried out at an initial pH of 4.7, gossypol inhibited but did not prevent hydrolysis. The results indicate that in an alkaline medium, gossypol in the presence of calcium produces a complete breakdown or precipitation of the enzyme-activator complex.

To determine whether or not calcium salts might be of value in counteracting the toxic effects of gossypol in the animal body, albino rats were fed acidic and basic diets containing 0.05 per cent gossypol and varying amounts of calcium. Favorable results, based on the number of animals which survived, their food intake, and gain in weight, were obtained with the basic diets containing excess (1.23 per cent) calcium. Further experiments dealing with the reaction between calcium and gossypol are discussed.

**THE FIBRINOLYTIC ENZYME OF HEMOLYTIC STREPTOCOCCI\***

BY R. L. GARNER

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The extracellular elaboration of the fibrinolytic enzyme<sup>12</sup> is a unique property of strains of hemolytic streptococci which are

\* Aided by a grant from the National Research Council.

<sup>12</sup> Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, **58**, 485 (1933). Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, **60**, 239, 255 (1934).

associated with human infections. By contrast, strains of hemolytic streptococci of animal origin (non-pathogenic for man) are usually devoid of this property. With respect to the intracellular proteinases, however, representative strains of the two types of organisms are shown to be identical.

The fibrinolytic enzyme is particularly characterized by its specificity. Although a variety of substrates have been tested, a detectable action is observed only with fibrin and fibrinogen isolated from human blood. The specificity of the reaction is further emphasized by the fact that preparations from the blood of lower animals are not susceptible to fibrinolysis.

The clotted plasma is rapidly and completely transformed into a limpid solution, while the fibrinogen solutions, after brief incubation with the enzyme, lose the capacity to form fibrin. Quantitative experiments are described which indicate that the dissolution of the fibrin is not accompanied by a marked chemical degradation of the protein molecule. Neither an evolution of ammonia nor an increase in the non-protein nitrogen can be detected. The fibrinogen is transformed into a substance (or substances) which retains characteristic protein properties but which may be differentiated from the parent fibrinogen by an increased solubility in concentrated salt solutions and by a higher temperature of denaturation.

By immunological experiments, the end-product of the reaction cannot be differentiated from the original fibrinogen solution, even though the two antigens possess different physical and chemical properties.

### IMMUNE GLOBULINS IN THE PLACENTA

BY ARDA ALDEN GREEN AND CHARLES F. MCKHANN

*(From the Department of Pediatrics, Harvard Medical School, Boston)*

Globulins separated from an extract of the human placenta carry antibodies for measles and poliomyelitis, scarlet fever, and diphtheria. The placental globulins may be divided into four fractions, depending on increasing solubility in salt. The identification of antibodies in these different fractions has been attempted. Whereas the virus antibodies are contained in most of the fractions, the antitoxic antibodies are more sharply localized.

**THE INFLUENCE OF CERTAIN LIVER POISONS ON THE ACTION OF  
PARATHYROID EXTRACT**

By DAVID M. GREENBERG

*(From the Division of Biochemistry, University of California Medical School,  
Berkeley)*

A study has been made of the effect of acute liver poisoning by phosphorus, carbon tetrachloride, and hydrazine sulfate on the response of the blood calcium of the dog to injections of parathyroid extract. Of these substances, phosphorus almost obliterated the rise in blood calcium which is normally obtained after parathyroid extract injection, hydrazine sulfate caused a small reduction in amount, while carbon tetrachloride appeared to have no retarding effect whatever.

To test the view-point that phosphorus might be producing its effect through an action on bone rather than the liver, the effect of benzene, known to be a bone marrow poison, was also determined. Acute intoxication with this substance produced no alteration in the response of the blood calcium level to parathyroid extract injection.

**THE EFFECT OF A DIET LOW IN MAGNESIUM ON THE RAT**

By DAVID M. GREENBERG AND ELMA V. TUFTS

*(From the Division of Biochemistry, University of California Medical School,  
Berkeley)*

In extending the work of Kruse, Orent, and McCollum<sup>13</sup> on the effect of a dietary magnesium deficiency, a diet containing between 1 and 2 mg. of magnesium per 100 gm. of dry food has been fed. The animals kept on this diet have never been observed to suffer spontaneous convulsions, but convulsive seizures can be induced through the application of a suitable stimulus, such as the hissing sound of an air blast. Moreover, when there was an ample quantity of vitamin G in the diet, at this level of magnesium, there did not develop the trophic changes, such as loss of hair, emaciation and edema of the feet, which are stated by Kruse, Orent, and McCollum to be characteristic of the terminal stages of magnesium deficiency.

<sup>13</sup> Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, **96**, 519 (1932); **100**, 603 (1933).

Chemically, in these animals, the total body magnesium was found to be reduced about 33 per cent and the plasma magnesium about the same amount below the values for the litter mate controls. The magnesium content of the brain, muscle, and liver of the deficient animals was not altered.

At a somewhat higher level of magnesium in the diet, namely 5 mg. of magnesium per 100 gm. of food, it has been possible to carry rats through pregnancy and to rear some of the young successfully through the period of lactation.

#### **ERRORS IN BIOASSAY: SOME UNUSUAL EFFECTS OF INORGANIC SALTS ON THE DETOXICATION OF BENZOIC ACID IN RATS**

By WENDELL H. GRIFFITH

*(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis)*

The determination of the extent to which young rats can detoxicate benzoate has demonstrated in a surprising way some of the difficulties in the use of the living organism as a "test-tube" for biochemical measurements. Survival, increase in weight, and the excretion of hippuric acid and of total combined benzoic acid were used as a measure of the detoxicating capacity of the animal. Toxic benzoate diets were made non-toxic by the addition of glycine or of proteins rich in glycine. It seemed probable, therefore, that the toxicity of a benzoate diet could be used as a means of bioassay for the determination of glycine or of its precursors. However, the interpretation of such experiments has been complicated by the results obtained following the addition of various inorganic salts to the food mixture. The effects produced by  $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ ,  $\text{NaCl}$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaHSO}_4$ , and the corresponding potassium salts could not be explained on the basis of the known action of these salts on the acid-base equilibrium. The fact that certain of the salts, such as  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{SO}_4$ , and  $\text{KHSO}_4$ , protected the animals from the toxicity of benzoate demonstrated that the detoxicating mechanism was subject to other factors in addition to the availability of glycine. The experiments emphasized in a striking manner the possible influence of supposedly unrelated factors on the metabolic reactions involved in any method of bioassay.

**THE MOLECULAR WEIGHT AND VOLUME OF HEMOGLOBIN IN UREA SOLUTIONS**

By DAVID B. HAND

*(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca)*

The molecular weight of hemoglobin is unaltered by the presence of urea up to concentrations as high as 6.66 M. The report in the literature that urea reduces the molecular weight of hemoglobin to 34,000 is explained by failure to account for the hydration of hemoglobin. The presence of urea increases the osmotic pressure of hemoglobin solutions, but this increase is quantitatively explained by increased hydration of the protein which, due to removal of the solvent, actually increases the concentration of the protein.

The apparent hydration of the hemoglobin can also be calculated by a number of empirical equations from the viscosity of the solution. The hydration calculated from the viscosity is about the same as that calculated from osmotic pressure for isoelectric hemoglobin in 0.5 M phosphate. But in urea solution the hydration calculated from osmotic pressure is much greater than that calculated from viscosity.

**AN INTERPRETATION OF THE DRIFT IN POTENTIAL IN THE QUINHYDRONE pH METHOD ON BLOOD OR BLOOD SERUM**

By MARTIN E. HANKE AND MARTHA JOHNSON

*(From the Department of Physiological Chemistry, the University of Chicago, Chicago)*

The nature of the drift in potential when quinhydrone is mixed with blood or blood serum is determined by the concentration of quinhydrone; high concentrations causing a positive drift, low concentrations, a negative drift. These facts are rationalized by the following theory, quinone + some reducing agent in serum  $\rightarrow$  hydroquinone + acid, and this reaction, 10 to 50 per cent complete in 5 or 10 minutes at room temperature, proceeds progressively with time. According to this theory, the positive factor is the formation of acid, the extent of which is greater as the quinhydrone concentration is increased; the negative factor is the reduction of the quinone to hydroquinone, which is more complete per unit time as the concentration of quinhydrone is decreased.

The following predictions of this theory have been tested and verified experimentally. (1) When quinone (not hydroquinone) is added to serum, *acid* is formed; at complete reaction, the amount of acid of 0.45 mole per mole of quinone. (2) The direction of the drift is eventually always negative. (3) When quinone is added to serum, it is completely converted into hydroquinone; hydroquinone does not react with serum. (4) The concentration of quinhydrone at which there is no drift in the first 5 or 10 minutes (positive and negative effects equally balanced) is determined by the buffer index of the serum.

Progress has been made in devising a procedure which eliminates the drift error as well as the drift itself and also the salt and protein errors of precise pH determinations by this method.

#### SALT AND WATER EXCHANGE BETWEEN BLOOD AND MUSCLE

By A. BAIRD HASTINGS AND LILLIAN EICHELBERGER

(From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago)

The purpose of the experiments to be described was to elucidate the exchange of water between blood and muscle. The data obtained have led the authors to the following conclusions.

(1) When muscle tissue is equilibrated *in vitro* with blood serum the cells die and become permeable to chlorides. (2) Muscle cells are ordinarily not permeable to chloride, sodium, or potassium. (3) Muscle cells normally contain no chloride and only approximately 5 mm of sodium per kilo of tissue. (4) All of the chloride and most of the sodium is present in the extracellular fluid of the tissue. (5) On the basis of these conclusions, the amount of extracellular fluid in muscle tissue has been calculated. (6) When acidosis is produced in an animal by rebreathing high concentrations of carbon dioxide, the amount of extracellular fluid decreases. (7) When a balanced isotonic salt solution is injected in large quantities intravenously, the amount of extracellular fluid increases without swelling or shrinking of the muscle cells. (8) When an acid isotonic salt solution is injected, the amount of extracellular fluid increases and the muscle cells swell slightly. (9) When an alkaline isotonic salt solution is injected, the amount of extracellular fluid increases greatly and the muscle cells swell appreciably. (10)

When hypertonic salt or sugar solutions are injected, the muscle cells shrink markedly and the amount of extracellular fluid remains constant or increases slightly.

**THE DEGREE OF UNSATURATION OF PHOSPHOLIPID FATTY ACIDS OF TUMOR TISSUE**

By FRANCES L. HAVEN

*(From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)*

The phospholipid fatty acids of rat carcino-sarcoma No. 256, from the Crocker Institute, were found to possess a low degree of unsaturation which is only slightly influenced by the nature of the dietary fat. Tumor tissue from animals raised on a fat-poor diet gives an iodine number of 85; coconut oil, 95; cod liver oil, 106; and menhaden oil, 113. Rat muscle values on the first three diets are respectively 100, 120, and 160. The location of the tumor does not affect the degree of unsaturation of its phospholipid fatty acids since subcutaneous, lung metastatic, and liver tumor fatty acids from animals raised on the same diet possess the same iodine number.

Since the views as to the function of phospholipids in normal tissue are still unsettled, one can speculate concerning their function in tumor tissue. (a) Highly unsaturated fatty acids may not be incorporated in the phospholipid molecule. Regarding phospholipids as cellular oxygen transport agents, the abnormal respiration of tumor tissue may be explained by the low degree of unsaturation of the fatty acids. (b) Considering phospholipids as intermediary products in fat metabolism, the highly unsaturated fatty acids may be built into the tumor phospholipids, then rapidly burned to supply energy to the tumor. (c) Tumor possesses no function other than growth. Therefore, unsaturated fatty acids may not be essential to tumor phospholipids as they are to muscle which must do external work. (d) Highly unsaturated fatty acids may be built into the phospholipids but immediately saturated owing to conditions within the tumor.

**VARIATIONS IN AMINO ACID CONTENT OF FINGER NAILS OF  
NORMAL AND ARTHRITIC INDIVIDUALS**

BY W. C. HESS

*(From the Chemo-Medical Research Institute, Georgetown University,  
Washington)*

It has previously been found that normal finger nails contain 6.60 per cent arginine, 0.46 per cent histidine, and 2.61 per cent lysine. These figures were obtained by the isolation procedures of Vickery and Block. Cystine in the same normal nails, by the Sullivan method, was 11.98 per cent, while by isolation only 9.57 per cent could be found. This work was repeated on 3.9 gm. of finger nails of persons suffering from arthritis. By isolation there was found 6.62 per cent arginine, 0.49 per cent histidine, and 2.63 per cent lysine. Cystine, determined by the Sullivan method, was 9.78 per cent. The arginine was also determined colorimetrically, in the arthritic nails, by a modified Sakaguchi method and histidine by the method of Kapeller-Adler. The two colorimetric methods gave figures of the same order as the isolation methods: 7.22 per cent arginine and 0.46 per cent histidine. The molecular ratios of histidine to lysine to arginine for the normal nails were 1:6:13, while for the arthritic nails they were 1:5.4:13.5. The three basic amino acids remained practically constant, while the cystine content decreased 18.4 per cent.

**THE IDENTITY OF THE ANTIDERMATITIS FACTOR**

BY ALBERT G. HOGAN AND LUTHER R. RICHARDSON

*(From the Department of Agricultural Chemistry, University of Missouri,  
Columbia)*

It was reported in an earlier paper that the type of dermatitis there described does not develop in rats when corn-starch is included in the diet. More recent studies have shown that the active agent of starch is easily extracted with hot alcohol. The starch residue neither heals the lesions nor prevents them from appearing. The ether extract of wheat germ has approximately the same potency as the starch extract. Mazola (corn oil) and commercial linseed oil were less active. Refined coconut oil and the ether extract of yeast have little or no activity. The effectiveness of the yeast was apparently undiminished by the extraction process.



Rats that have been healed of dermatitis by the starch extract, 100 mg. daily, make little or no growth and if they survive long enough the hair becomes thin within 12 to 16 weeks. In severe cases they develop the denuded condition described by Sherman and his collaborators as characteristic of a deficiency of vitamin G ( $B_2$ ). As to the identity of the antidermatitis factor, the available evidence indicates that it is a distinct entity and should be given a separate classification.

#### CHEMICAL STUDIES ON TOAD POISONS: *BUFO ARENARUM*, *BUFO REGULARIS*, AND *XENOPUS LAEVIS*

By H. JENSEN

(From the Laboratory for Endocrine Research, the Johns Hopkins University, School of Medicine, Baltimore)

Arenobufagin  $C_{25}H_{34}O_6$  (secretion of *Bufo arenarum*) and regularobufagin  $C_{25}H_{34}O_6$  (secretion of the South African toad *Bufo regularis*) have been prepared and found to be isomers; both are lactones. The analytical data obtained from various derivatives of these bufagins substantiate the empirical formulas assigned to them. Like the bufagins obtained from the secretions of other species of toads, arenobufagin and regularobufagin are  $C_{23}$  derivatives (after removal of the acetyl radical which is attached to a hydroxy group) and show marked resemblance in chemical behavior to that of the aglucones of certain plant glucosides, which are also  $C_{23}$  derivatives. Epinephrine has been isolated and identified as such from the secretions of both *Bufo arenarum* and *Bufo regularis*. From the skin secretion of the South African toad *Xenopus laevis* a base (obtained as a flavianate) has been isolated and was found to be identical with bufotenidine. No evidence has been obtained so far of the presence of any bufagin-like substance in this secretion.

#### FURTHER CHEMICAL INVESTIGATION OF CRYSTALLINE INSULIN

By H. JENSEN, E. A. EVANS, JR., W. D. PENNINGTON,  
AND ELLEN D. SCHOCK

(From the Laboratory for Endocrine Research, the Johns Hopkins University, School of Medicine, Baltimore)

The behavior of cystine and the free amino nitrogen of insulin under the action of various reagents which inactivate the hormone,

either reversibly or irreversibly, has been investigated. The following reagents have been employed:  $n/30$  NaOH, formaldehyde, benzaldehyde, *o*-chlorobenzaldehyde, acetic anhydride, methyl iodide, hydriodic acid, iodine, HCl and methyl alcohol, diazomethane, and nitrous acid.

It was further found jointly with Dr. L. Hellerman (Department of Physiological Chemistry) that insulin was inactivated when treated with benzoquinone (in  $m/15$   $Na_2HPO_4$  solution in a nitrogen atmosphere). In acid solution no inactivation occurs. Hydroquinone does not influence the activity under the same conditions. Unlike cysteine and glutathione, the thiol compounds thiohistidine and ergothionine do not readily inactivate insulin; thiolsalicylic acid under the same conditions renders the hormone inactive. The inactivation of insulin by the different reagents may be explained as the result of either destruction or modification of certain component groupings in the molecule.

#### A COMPARISON OF THE DISSIPATION OF HEAT MEASURED BY THE INSENSIBLE LOSS OF WATER WITH THE HEAT PRODUCTION DETERMINED BY INDIRECT CALORIMETRY FOR PERIODS OF TWENTY-FOUR HOURS

BY MARGARET WOODWELL JOHNSON AND L. H. NEWBURGH

*(From the Department of Internal Medicine, University of Michigan, Ann Arbor)*

The subjects were fed constant diets of known composition during the experiment and for at least the 3 preceding days. They entered the respiration chamber of the open circuit type at night. Starting under basal conditions the next morning, the following data were obtained for the next 24 hours: (1) insensible loss of weight; (2) oxygen absorption; (3) carbon dioxide production.

The heat production was calculated by standard methods. The insensible loss of water was calculated from the equation  $IW = IL - (CO_2 - O_2)$ , where  $IW$  is insensible water and  $IL$  is insensible loss of weight. The heat lost by the vaporation of water equals  $IW \times 0.58$  and the total heat elimination is assumed to be  $IW \times 0.58 \times (100/24)$ . This value was compared with the heat production obtained by indirect calorimetry.

**FURTHER OBSERVATIONS ON THE POSSIBLE INTERRELATIONSHIP IN THE PHYSIOLOGICAL ACTION OF THE PARATHYROID GLANDS AND VITAMIN D**

BY JAMES H. JONES

*(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)*

Young pups, given a modification of the Cowgill<sup>14</sup> diet to which had been added 0.75 per cent of beryllium carbonate, developed marked clinical rickets in a few weeks. Ultra-violet irradiation or cod liver oil failed to prevent the development of the rachitic manifestations. These animals were practically immune to the action of large doses of parathyroid extract, even though a liberal amount of vitamin D was administered. Pups which were made rachitic on the Be-containing diet without vitamin D likewise gave only a slight response to parathyroid extract. Ultra-violet irradiation of these animals for several weeks failed to increase the response.

Although showing only a slight reaction to parathyroid extract, these animals developed typical symptoms of hypervitaminosis when 150,000 international units of irradiated ergosterol per kilo of body weight were administered daily. The response was only slightly less than that of normal controls. Since parathyroid extract was inactive for rachitic animals, although vitamin D was present, it appears that the failure was not due to a lack of vitamin stores in the body but to some other factor. A lack of available calcium has been suggested by other workers. However, since these animals which did not react to parathyroid extract readily developed toxic symptoms when large doses of irradiated ergosterol were administered, it is highly improbable that the toxicity was due to a stimulation of the parathyroid glands.

<sup>14</sup> The diet was composed of the following ingredients, expressed in per cent: casein 37.6, sucrose 32.9, lard 19.0, salt mixture (Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1933)) 2, yeast 5, bone ash 0.5, agar 1.5, butter fat 1.5.

**THE SUBSTITUTION OF CERTAIN CYSTINE DERIVATIVES FOR  
CYSTINE IN THE GROWTH OF RATS**

By JAMES H. JONES, KATHLEEN CRANDALL ANDREWS, AND  
JAMES C. ANDREWS

*(From the Department of Physiological Chemistry, School of Medicine,  
University of Pennsylvania, Philadelphia)*

We have tested the ability of the following compounds to substitute for cystine in the diet of growing rats: cystine hydantoin, cystine phenylhydantoin, cysteic acid hydantoin, cysteic acid phenylhydantoin, and dibenzoylcystine. These compounds were fed at levels equivalent in sulfur content to 0.05 per cent cystine. With none of the above compounds, except dibenzoylcystine, have we observed any increase in weight when the diets were fed over a period of 7 weeks or more.

In the case of dibenzoylcystine the rate of growth, while not duplicating that resulting from administration of cystine, showed occasional irregular increases above that of the control animals. This finding harmonizes with the results obtained in this laboratory and by other investigators on the metabolism of dibenzoylcystine which, when fed or administered by way of an isolated intestinal loop, is partly oxidized to inorganic sulfate. These results have been interpreted as indicating partial hydrolysis to free cystine which, in the present experiments, would result in a greater growth increment.

**MEASUREMENT OF TRYPTIC DIGESTION BY DIRECT TITRATION**

By THOMAS H. JUKES

*(From the Division of Poultry Husbandry, University of California, Davis)*

Proteins were digested with a commercial trypsin preparation. The digest, containing phenolphthalein, was first adjusted to pH 8.5. A rapid liberation of acid was found to take place. At intervals, aliquots were withdrawn and titrated back to pH 8.5 with sodium hydroxide. Simultaneously, the rupture of peptide linkages was measured by formol titration. It was found that the production of acidity closely paralleled formol titration value and hence that direct titration with alkali provided a rapid and convenient measurement of protein hydrolysis.

The explanation of the liberation of acidity is to be found in the

fact that only in the isoelectric zone is rupture of the peptide linkage unaccompanied by change of pH. At the pH of optimal peptic or tryptic activity, proteolysis is accompanied by an increase in buffering power which tends to shift the pH towards the isoelectric point of the peptide mixture.

In the case of certain proteins, such as casein, lecitho-vitellin, and ovomucoid, which contain esterified prosthetic groups, an unusually large production of acidity was observed to take place during the initial stages of digestion. This was thought to be due to hydrolysis of the ester linkage. Support is lent to this view by the observation that acid-soluble phosphorus is liberated more rapidly than acid-soluble nitrogen during tryptic digestion of casein and lecitho-vitellin.

#### THE DECOMPOSITION OF CAROTENE DURING THE STORAGE OF HAYS AND MEALS

By EDWARD A. KANE AND LEO A. SHINN

*(From the Research Laboratories, Bureau of Dairy Industry, United States  
Department of Agriculture, Beltsville, Maryland)*

Recent work on the effect of rations low in vitamin A upon reproduction in cattle and upon the vitamin A potency of their milk has made it very desirable to know more about the carotene content of their feeds. Work has been reported from this laboratory showing that frequently only 10 per cent as much carotene occurs in market hays as in the fresh green material from which they are made; and that large losses of carotene occur during the storage of hays, especially in warm weather. We have now studied the losses of carotene occurring during the storage of alfalfa meals. Meals, ground to  $\frac{3}{4}$  inch,  $\frac{1}{4}$  inch, and  $\frac{1}{8}$  inch mesh, were prepared from the same bale of hay and stored under conditions similar to those in a dark hay barn. Meals from four lots of hay were prepared in this way and stored for periods of 7 to 8 months beginning in March. The average loss of carotene with the meals that were ground to a  $\frac{3}{4}$  inch mesh was 40.4 per cent, to a  $\frac{1}{4}$  inch mesh 36 per cent, and to a  $\frac{1}{8}$  inch mesh 36.5 per cent. The loss of green color in these meals during this period was determined by the Hay Office of the Bureau of Agricultural Economics. These losses were 16.3, 10.9, and 12.8 per cent respectively. The losses of carotene in

these meals were practically the same as in samples from the same lots of hay that were stored in bales.

## THE DETERMINATION OF CYSTINE AND RELATED DISULFIDES WITH THE PULFRICH PHOTOMETER

By BEATRICE KASSELL

(From the Department of Chemistry, New York State Psychiatric Institute  
and Hospital, New York)

Parts of Lugg's<sup>15</sup> modification of the Folin and Marenzi<sup>16</sup> method were used in developing a procedure to determine cystine and related sulfur compounds.

Filter S-72 was selected; the calibration curve indicates a linear relationship between the extinction coefficient and the concentration of cystine from 0.1 to 3 mg. per cent. The extinction coefficient of 1 mg. per cent of cystine, 0.343, did not vary with different preparations of the color reagent if made by the same technique.<sup>17</sup>

In the determination, Solution A<sup>17,a</sup> is prepared according to the directions of Lugg,<sup>15</sup> except that double the amount of color reagent is used. If interfering substances are present, Solution A is read in the photometer, first against Solution B and second against Solution C. The average of these two readings gives the cystine concentration. If the extinction coefficient of Solution C is less than 0.027, then the reading of Solution A against C is omitted and the value for cystine obtained directly.

When cysteine is also present, a fourth solution, D, is needed. Cysteine is determined by reading Solution C against D. In this case, the value for cystine is obtained by reading Solution A against B and D, averaging these values, and subtracting twice the amount of the cysteine determined as above.

Homologous disulfide compounds<sup>18</sup> show a rate of color development which decreases with increasing molecular weight. The rate of color development may be accelerated by increasing the amount of sulfite, thus permitting the determination of any one of these disulfides in the presence of another.

<sup>15</sup> Lugg, J. W. H., *Biochem. J.*, **26**, 2144, 2160 (1932).

<sup>16</sup> Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).

<sup>17</sup> Folin, O., *J. Biol. Chem.*, **101**, 111 (1933).

<sup>17,a</sup> Solution A, cystine or unknown, color reagent, buffer, and sulfite; Solution B, A + HgCl<sub>2</sub>; Solution C, A without sulfite; Solution D, C + HgCl<sub>2</sub>.

<sup>18</sup> We are indebted to Dr. V. du Vigneaud for the pento- and hexocystine used in these experiments.

**THE CHEMICAL NATURE OF CORTIN**

By EDWARD C. KENDALL, HAROLD L. MASON, BERNARD F. MCKENZIE, AND CHARLES S. MYERS

*(From the Division of Chemistry, The Mayo Foundation, Rochester, Minnesota)*

Crystals will separate from partially purified cortin treated with a small volume of water and ether. Purification gives a crystalline compound which is oxidized to an acid without loss of carbon with ammoniacal silver. Oxidation of the acid with chromic acid results in the loss of 2 atoms of carbon and the formation of a ketone.

The most probable structure is a trihydroxy aldehyde. Two hydroxyl groups are in positions  $\alpha$  and  $\beta$  to the aldehyde. The compound is therefore a derivative of glyceraldehyde. With hot sodium hydroxide the aldehyde is converted into an acid with intramolecular rearrangement. Absence of a specific absorption spectrum shows the absence of the benzene ring. Distillation of the ketone with zinc dust gives a hydrocarbon of high molecular weight, apparently three 6-membered rings fully saturated.

Oxidation with silver of solutions from which the crystals separate gives products similar to the acid and ketone, except that they are more unsaturated. The specific rotation of the crystalline material is from  $65^\circ$  to  $110^\circ$ . The specific rotation of products from the mother liquor of the crystals is as high as  $180^\circ$ .

**THE VITAMIN B COMPLEX AND NUTRITIONAL ANEMIA IN THE WHITE RAT**

By JEAN L. KYER AND FRANK H. BETHELL

*(From the Thomas Henry Simpson Memorial Institute for Medical Research, Ann Arbor)*

During the course of some studies on the prevention and cure of milk anemia in the white rat and the regeneration of blood with iron and copper supplements, it was observed that another substance, probably organic, played a very important rôle.

Both in prevention and curative experiments the rats were placed on a basal diet of reconstituted dry milk of known iron and copper content. Rats receiving 26.6 per cent reconstituted milk instead of the customary 14.7 per cent milk failed to develop a severe anemia. When the iron and copper content of 14.7 per

cent reconstituted milk was increased to the quantities ingested by rats receiving the 26.6 per cent milk, severe anemia was not prevented.

During the investigation of various substances present in milk which might account for its hematinic action, a 70 per cent alcoholic extract of dried brewers' yeast was employed. The yeast extract exerted a definite influence upon both the prevention and cure of anemia. As the iron and copper content of the very small amount of yeast extract used could not account for its effect, the study of the influence of the vitamin B complex was undertaken. Neither vitamin B<sub>1</sub> nor B<sub>2</sub> seemed to have any effect, but the vitamin B<sub>4</sub> fraction exerted the same influence as the whole yeast extract.

The vitamin B<sub>4</sub> fraction apparently stimulates erythropoietic activity in the white rat, possibly by its influence on hemoglobin synthesis.

#### THE EFFECT OF MALNUTRITION ON THE PATHOGENESIS OF RAT LEPROSY

By ALVIN R. LAMB

*(From the United States Leprosy Investigation Station, Honolulu, Hawaii)*

Rat leprosy is caused by an organism morphologically and tinctorially identical with the bacillus of human leprosy, and the disease is pathologically very similar to human leprosy. Rats inoculated subcutaneously with rat leprosy were subjected to many dietary deficiencies, singly and in combination. Whenever the effects of the dietary deficiencies were complicated by inanition, results were generally negative. A diet of starchy foods plus taro root and fish, less severely deficient in the vitamin B complex and in calcium, repeatedly increased the development of subcutaneous lesions. Diets deficient in the vitamin B complex, which permitted suboptimum reproduction, produced a similar increase in the lesions in the third and fourth generations.

When intracardiac inoculations were made, diets deficient in the vitamin B complex and somewhat low in protein produced an extensive increase in visceral lesions of rat leprosy. The liver was the principal site, and showed very large and confluent lesions in the experimental animals. The spleen, lungs, and lymph nodes



were involved, and the skin was generally infiltrated with organisms and contained microscopic lesions of rat leprosy. Small lesions occurred in the same sites in the control rats, but were almost always sharply limited in size on the normal diet. Similar marked increases in leprous pathology were found after intra-cardiac inoculation of fourth generation rats on a diet less severely deficient in the vitamin B complex.

#### COMPARISON OF THE XYLOSE TOLERANCE WITH BLOOD UREA IN NEPHRITIC RATS

By HARDY W. LARSON

*(From the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York)*

The clearance of xylose, used as a measure of renal function, is believed to give results comparable with the urea clearance. Some think that renal injury should be reflected in a slight retention of urea which would be recognizable provided the range of the normal urea is sufficiently established. This study was planned to obtain information on these points. One pole of the kidney was ligated to cause degeneration beyond the ligature and 2 weeks later the other kidney was removed. The rats were then fed a diet known to produce renal injury. 1 month later the animals were given xylose by stomach tube and the blood xylose was followed for 5 hours. Blood urea was determined at the same time. This procedure was repeated at monthly intervals until either the xylose curve became abnormal or there was retention of urea. The rats were then sacrificed and the kidneys examined for pathological changes. The results showed that either the xylose tolerance might become abnormal or urea might be retained without evidence of marked kidney involvement.

#### NOTE ON THE INSULAR HORMONE

By NELLES B. LAUGHTON AND A. BRUCE MACALLUM

*(From the Physiological and Biochemical Departments, Faculty of Medicine, University of Western Ontario, London, Canada)*

The preparation reported by us<sup>19</sup> has been fractionated and prepared in a more concentrated form. While the original prepara-

<sup>19</sup> Laughton, N. B., and Macallum, A. B., *Proc. Roy. Soc. London, Series B*, 111, 37 (1932).

tion will not reduce an experimental hyperglycemia in normal rabbits below the normal level, the concentrated fraction will reduce such a hyperglycemia below 100 mg. of sugar per 100 cc. to levels as low as 65 mg. These, however, revert rapidly to the normal level without producing any symptoms.

The method described<sup>19</sup> has been found to yield a preparation from the gastric mucosa and liver tissue which has physiological effects identical with that from the duodenal mucosa.

### A DISULFOXIDE OF L-CYSTINE

BY THEODORE F. LAVINE AND GERRIT TOENNIES

*(From the Lankenau Hospital Research Institute, Philadelphia)*

The oxidation of solutions of cystine perchlorate in acetonitrile by means of perbenzoic acid was studied at various temperatures and the optimum conditions for oxidation of the cystine to a level corresponding to the disulfoxide established. The relatively slight solubility of the disulfoxide in water was made the basis of its separation from other oxidation products. The ultimate analysis and level of oxidation of the isolated material corresponded to the requirements of the disulfoxide. Its decomposition point, optical rotation, isoelectric point, and solubility were determined.

The oxidizing action of the disulfoxide on aqueous solutions of HCl and KI was developed into a quantitative method for the estimation of "intermediate oxygen."

By virtue of its intermediate level of oxidation, the disulfoxide in aqueous solution undergoes dismutative decomposition; in alkaline solution this decomposition proceeds rapidly according to the equation  $3\text{R}(-\text{S}-\text{O})_2\text{R} + 2\text{H}_2\text{O} \rightarrow \text{R}-\text{S}-\text{S}-\text{R} + 4\text{R}-\text{S}-\text{O}_2\text{H}$ . Although the same general decomposition slowly takes place in acid solutions, the results are not as definite as in the case of the alkaline solutions.

Cysteine reacts practically instantaneously with the disulfoxide in either acid or alkaline solution, according to the reaction,  $\text{R}(-\text{S}-\text{O})_2\text{R} + \text{R}-\text{S}-\text{H} \rightarrow \text{R}-\text{S}-\text{S}-\text{R} + \text{R}-\text{S}-\text{O}_2\text{H}$ . When excess cysteine is present, the sulfinic acid slowly reacts to form cystine,  $\text{R}-\text{S}-\text{O}_2\text{H} + 3\text{R}-\text{S}-\text{H} \rightarrow 2\text{R}-\text{S}-\text{S}-\text{R} + 2\text{H}_2\text{O}$ .

**THE PREPARATION OF CRYSTALLINE VITAMIN G**

BY SAMUEL LEPKOVSKY, WILLIAM POPPER, JR., AND  
HERBERT M. EVANS

(From the Institute of Experimental Biology, University of California,  
Berkeley)

Vitamin G can be easily prepared in crystalline form by the following method. An extract of liver is shaken with fullers' earth, and the activated clay eluted with dilute sodium hydroxide. The solution is neutralized with hydrochloric acid and again adsorbed with fullers' earth. The fullers' earth is again eluted with dilute sodium hydroxide, and the eluate neutralized with hydrochloric acid and concentrated *in vacuo*. It is centrifuged occasionally during the concentration, and when quite concentrated, the vitamin G drops out as a very fine crystalline mass. It is recrystallized a few times from hot water and dehydrated with acetone. It is a dark yellow non-hygroscopic powder. 0.1 mg. enabled rats on a vitamin G-free diet to gain about 26 gm., and 0.3 mg. about 30 gm. in 9 days.

**THE CONCENTRATION AND PURIFICATION OF THE  
GONADOTROPIC SUBSTANCE OF THE URINE OF  
HUMAN FEMALE CASTRATES**

BY LOUIS LEVIN\* AND H. H. TYNDALE

(From the Department of Anatomy, College of Physicians and Surgeons,  
Columbia University, New York)

Methods described in the literature for the concentration of the gonadotropic material of human pregnancy urine did not yield equally satisfactory results when applied to the urine of human female castrates. A method depending on tannic acid precipitation was found to be an effective means of concentrating all or nearly all the gonadotropic activity of these castrate urines. Alkaline extracts of the acetone-washed precipitates are potent and non-toxic to immature mice, even if administered in doses representing large quantities of urine, the maximum tested to date being 600 cc.

Barium precipitation of such extracts results in considerable purification, but appreciable activity is lost. Marked purification,

\* Squibb Fellow in Anatomy, 1934-35.

with little loss of activity, is effected by leaching the dry tannate powder with 70 per cent ethyl alcohol (solution discarded). The active principle is then dissolved in aqueous acetone (or alcohol) containing ammonia and reprecipitated by increasing the acetone concentration. These precipitates contain all or nearly all the activity of the original tannate but only 30 to 35 per cent of the original solids. They are readily water-soluble and may be administered in doses representing very large quantities of raw urine.

Immature mice and rats were used for the assays. A combination of four factors (ovarian weight, uterine weight, precocious vaginal opening, and microscopic appearance of the ovaries) was used as the criterion of gonadal stimulation. Histological examination of certain selected ovaries was made.

This method removed 70 to 80 mouse units of gonadotropic material per liter from pooled specimens of urine of human female castrates.

## STUDIES IN CYSTINURIA

By HOWARD B. LEWIS

*(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)*

Oral administration of *dl*-methionine (3.0 to 3.6 gm.) to a young cystinuric patient resulted in an increased excretion of cystine. The amount of extra cystine excreted after administration of methionine was greater when the diet contained moderate amounts of protein (urinary nitrogen, 7.5 to 8.5 gm. daily) than when large quantities of protein were fed (urinary nitrogen, 15.0 gm. daily). On the lower level of dietary protein, the excretion of extra cystine continued for several days after the methionine was fed, while on the higher protein diet, little extra cystine was excreted except on the days on which the methionine was fed. No extra cystine was excreted after the feeding of cystine.

The cystine content of the hair of cystinurics of two family groups has been studied. It was thought that any cystine deficiency in the hair would be more apparent in this group of young cystinurics, where the demand for sulfur compounds for growth was greater than in adults. The cystine content of the hair of three cystinuric children, 5 to 10 years of age, did not differ mate-

rially from that of their normal brothers or sisters of approximately the same age. Analyses of the hair of adult cystinurics also showed no abnormalities in cystine content.

#### ABSORPTION SPECTRA OF INDIGO SULFONATES

By ROBERT G. LOEFFEL

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis)

The extinction coefficients of spectra recorded by Holmes<sup>20</sup> for mono-, di-, tri-, and tetrasulfonates of indigo are found to be lower than the correct values, apparently owing to inadequate drying of Holmes' preparations. The peaks of the bands given by Holmes are approximately correct. The spectra of the dyes (as oxidants) have been measured also in highly alkaline solutions.

The *leuco* form of each dye exhibits *three* different colors, each characterizing a different pH zone. This fact is evidence of *two* acidic groups, the dissociation constants of which have been approximately determined from the color change. Sullivan, Cohen, and Clark were unable to detect (by titration) the second acidic groups in the leuco forms. The spectra of the leuco forms in solutions of high pH are distinctive and permit identification among the four sulfonates.

The cherry-red intermediates between the blue oxidant and the yellow leuco forms, pH 11 to 12, are distinctive and exhibit bands, the location of which permits ready identification among the four sulfonates, the peaks being located approximately as follows: mono- 548; di- 553; tri- 577; tetra- 590  $\mu$ .

#### OBSERVATIONS ON ADRENALECTOMIZED-DEPANCREATIZED AND HYPOPHYSECTOMIZED-DEPANCREATIZED CATS

By C. N. H. LONG AND F. D. W. LUKENS

(From the George S. Coz Medical Research Institute, University of Pennsylvania, Philadelphia)

The material for this study consisted of sixteen depancreatized cats (Group A), fifteen adrenalectomized-depancreatized cats (Group B), and six hypophysectomized-depancreatized cats (Group C).

<sup>20</sup> Holmes, W. C., *J. Am. Chem. Soc.*, **46**, 208 (1924).

The adrenalectomized animals received varying amounts of a commercial cortical extract, but all of them sooner or later showed mild or marked symptoms of cortical insufficiency. As a consequence the survival and behavior of adrenalectomized-depancreatized animals adequately treated with cortical hormone remain an unsettled question.

The average survival of Group A was 4 days (range 2 to 8 days), of Group B 10 days (range 4 to 28 days), and of Group C 40 days (range 18 to 85 days). None of the animals in Groups B and C died in coma, while in Group A this was the mode of death in fourteen out of sixteen cats.

Under fasting conditions the average urinary glucose of Group A was 3.9 gm. per kilo a day, of Group B 0.4, and of Group C 1.7. The corresponding urinary nitrogen excretions were 1.6, 0.6, and 1.0 gm. per kilo per day.

In contrast to the doubly operated animals (Groups B and C), the majority of depancreatized cats exhibits marked ketonuria 2 to 3 days after operation. In the former the excretion of acetone bodies is within normal limits throughout life, unless steps are taken to induce it.

The injection of anterior pituitary extract (Squibb) did not increase the glycosuria or ketonuria in the adrenalectomized-depancreatized animals but had a well marked effect on the hypophysectomized-depancreatized ones. Epinephrine increased the glycosuria in most of the doubly operated animals but only occasionally increased the ketonuria.

#### **GALACTOSE TOLERANCE AS MEASURED BY THE FOLIN MICRO AND MACRO BLOOD SUGAR METHODS**

BY JOSEPH M. LOONEY AND E. MORTON JELLINEK

*(From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester)*

Sugar determinations were made by the Folin micro- and macro-methods on blood taken simultaneously, at 30 minute intervals, from the ear and vein of twenty-five schizophrenic patients who took 40 gm. of galactose.

In blood from the ear the microvalue is consistently higher than the macrovalue in venous blood, giving a fasting blood sugar level

of 97.5 mg., a maximum at 1 hour of 122 mg., and after 2 hours 95.0 mg. The macrovalues are control, 82 mg., 110 mg. at 1 hour, and 88 mg. at 4 hours.

The correlation coefficients between the micro- and macro-methods are for control, 0.16; at 1 hour, 0.67; at 3 hours, 0.20. The methods give a correlation of 0.62 for identical blood samples taken simultaneously from artery and vein. Then the micro mean value is consistently 6 mg. lower than the macro, and the arterial value is 3 mg. higher than the venous by either method. The values for arterial blood are micro 86.3 mg., macro 92.7 mg., for venous blood, micro 83.5 mg., macro 89.6 mg. The mean for arterial blood by the micromethod is 11 mg. lower than that from the ear, which indicates that tissue fluids squeezed out by manipulating the ear cause increase above the true value and make the results unreliable.

The micromethod is less reliable than the macromethod, as it gives a correlation of only 0.20 between arterial and venous sugar, while the latter gives one of 0.64.

#### THE OVARIAN FOLLICULAR HORMONE

By D. W. MACCORQUODALE, SIDNEY A. THAYER, AND  
EDWARD A. DOISY

*(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis)*

An attempt to isolate the estrogenic compound of liquor folliculi has given some interesting information.

Several crystalline preparations which seem to be identical with each other have been obtained. The potency of these products in spayed rats is about 6 times that of theelin.

The distribution ratio between 70 per cent ethyl alcohol and benzene is approximately 1; for theelin,  $\frac{1}{3}$ ; for theelol, 5.

The crystalline material is soluble in dilute alkali, ether, ethyl alcohol, and benzene. The solubility in petroleum ether or water is very low.

The crystals sublime at 0.02 mm. and 100°.

The hormone is readily extracted from some organic solvents with 0.2 N NaOH; it can be removed from the aqueous alkali by extraction with ether.

**OBSERVATIONS ON THE EXCRETION OF ESTRIN DURING PREGNANCY**

BY G. F. MARRIAN, S. L. COHEN, AND M. WATSON

*(From the Department of Biochemistry, University of Toronto,  
Toronto, Canada)*

With the colorimetric method of assay previously developed by Cohen and Marrian, a quantitative study of the "free" (ether-soluble and physiologically active) and "combined" (ether-insoluble and relatively physiologically inactive) estrone and estriol present in human urine at different stages of pregnancy has been made. The combined estrone and estriol show a rapid increase from about the 6th month onwards, reaching figures averaging 3.0 and 22.0 mg. per 24 hours respectively at 8½ months. Up to this time the amounts of free estrone and estriol are 1 per cent or less of the amounts of these substances in the combined forms. Shortly before and during labor there occurs a sharp fall in the amounts of combined estrone and estriol excreted, and simultaneously the amounts of the free hormones in the urine begin to rise.

24 hours after parturition the amounts of the free hormones in the urine are greatly in excess of the amounts of the combined forms. 48 hours after, the amounts of both forms have fallen to a very low level.

Changes in the estrin excretion similar to those observed before and during normal labor have been observed to occur in pseudo-labor.

The results obtained may possibly be considered to lend some support to the view that estrin is concerned in the parturition mechanism.

**SOME EFFECTS OF THE INTRODUCTION OF ELECTROLYTES INTO THE CISTERNA ON THE BLOOD PRESSURE OF DOGS**

BY M. F. MASON, H. RESNIK, JR., AND TINSLEY R. HARRISON

*(From the Departments of Biochemistry and Medicine, Vanderbilt  
University School of Medicine, Nashville)*

Observations have been made concerning the response of blood pressure to variations in the composition of the cerebrospinal fluid.



Various salts were introduced into the cisterna magna of dogs and changes in blood pressure were recorded. It was found that potassium, ammonium, and phosphate ions in small concentrations produced well marked elevation in blood pressure. Whereas the calcium *per se* had little effect on the blood pressure, it could inhibit the rise in blood pressure ordinarily resulting from the introduction of pressor salts.

These studies are being correlated with observations on the electrolyte pattern of the cerebrospinal fluid of patients with hypertension.

#### EFFECT OF DEXTROSE INGESTION ON SERUM INORGANIC SULFATE

By M. R. MATTICE, MAURICE BRUGER, AND M. DEREN

(From the Departments of Biochemistry and Medicine, New York Post-Graduate Medical School and Hospital, New York)

The inorganic sulfate content of the serum (method of Power and Wakefield<sup>21</sup>) was studied in twenty-three subjects before and at intervals of 30 minutes, 1 hour, and 2 hours following the ingestion of 100 gm. of dextrose. Four types of curves were obtained; *viz.*, (1) a gradual and persistent decrease (seven cases), (2) a preliminary fall at the 30 minute period followed by a rise to the control level (six cases), (3) a preliminary increase at the 30 minute period with a subsequent fall below the control level (seven cases), (4) a maintained rise throughout the test (three cases). The average of all figures for inorganic sulfate showed a diminution from the control approximately 0.1 mg. per cent at the 30 minute period, 0.4 mg. per cent at the 1 hour period, and 0.7 mg. per cent at the 2 hour period.

In a second series, the serum inorganic sulfate was studied in eleven subjects before and at 30 minutes following each of two doses of 50 gm. of dextrose given 30 minutes apart. The sulfate changes were similar to those described above except that the variations were less marked.

No direct relationship was observed between the changes in the whole blood sugar and the serum inorganic sulfate, although there

<sup>21</sup> Power, M. H., and Wakefield, E. G., *Proc. Staff Meetings Mayo Clin.*, **6**, 401 (1931).

was a general tendency for the sulfate to fall as the blood sugar increased.

### STUDIES OF FAT METABOLISM IN LACTATION

BY L. A. MAYNARD AND C. M. McCAY

*(From the Laboratory of Animal Nutrition, Cornell University, Ithaca)*

Studies in this laboratory and elsewhere have failed to confirm the view that phospholipid is the blood precursor of milk fat. Our results show, however, that some blood lipid is taken up by the mammary gland. The problem has been studied further with cows and goats by following the changes in the iodine numbers of the blood and milk lipids, which result from feeding saturated and unsaturated fats. A change in the iodine number of the food fat is reflected in a marked corresponding change in the milk fat, usually within 24 and always within 48 hours, and the full effect is generally reached in 96 hours. This is a surprisingly quick response in view of the nature of digestion in the ruminant and in view of certain current ideas on fat secretion. The changes in the iodine numbers of the total lipids of the blood are small compared to the corresponding changes in the milk. The much higher degree of saturation of the milk fat than of any of the lipid fractions of the blood means either a highly selective action by the gland or extensive transformations within the gland itself. Studies of the iodine numbers of the total lipids of the jugular and mammary vein blood fail to indicate a highly selective action, although the changes are in general in the direction expected. Some preliminary evidence has been obtained that the cholesterol esters may be concerned in fat secretion.

### HOMOZYGOUS MICE AS BIOCHEMICAL TEST ANIMALS

BY J. F. McCLENDON AND HAROLD STREET

*(From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis)*

Although the Wistar rat is a classical biochemical test animal, where only small amounts of substances are available it is desirable to use mice. Mice are quite hardy and resist extreme cold if free from infections. The objection raised to mice on account of slow growth rate is not valid unless the mice show greater

variability. This variability is less the more homozygous the breed of mice in regard to genes influencing growth. Bagg albinos have been used by a number of investigators. The coefficient of variability in body weight at birth is 15 per cent, at 1 week about 18 per cent, at 2 weeks 20 per cent, at 3 weeks 21 per cent; then the variability decreases so that at 8 weeks it is about the same as at birth with but slight sex difference. This is somewhat greater variability than in rats but not sufficient to warrant the use of rats for very expensive substances. Bagg albinos grow about 0.3 gm. per day to about the 18th day, when they almost cease growing; they then recover their former growth rate, which the males maintain up to the 8th week but which is gradually reduced in the females. Rickets is as easily produced in these mice and as easily detected by means of x-rays as in rats. The mice are useful in testing vitamins, hormones, and amino acids. The breed of mice makes a difference in growth studies, as the mean body weight at 4 weeks of age is 9.64 gm., whereas that of other breeds of white mice varies from 8.55 to 12.07 gm.

#### **A BIOCHEMICAL METHOD FOR THE ASSAY OF THE THYROTROPIC HORMONE OF THE PITUITARY GLAND**

By D. ROY McCULLAGH AND BENJAMIN F. STIMMEL

*(From the Department of Biochemical Research, Cleveland Clinic, Cleveland)*

The histological appearance of the thyroid gland in animals varies so greatly that the value of such microscopical studies for the assay of thyrotropic hormone of the pituitary gland is quite limited. The change in the basal metabolic rate of small animals treated with thyrotropic hormone supplies an accurate method of assay. However, equipment for accurate determination of the basal metabolic rate of animals is not available in most laboratories. For these reasons, we have used the decrease in iodine content of the thyroid gland in guinea pigs as a method of assay. 1 unit of thyrotropic hormone is defined as that amount of hormone which when injected daily for 3 days into a guinea pig weighing 300 gm. will cause a 50 per cent decrease in the thyroid iodine. The normal figure for thyroid iodine is established by analyses of thyroid glands of at least six animals raised on the same diet and under the same conditions as the group used for assay. Animals are injected in groups of four, the groups receiving varying doses

to determine the exact potency of the preparation. The iodine determinations are carried out by the method of the senior author. It is possible to make from sixteen to twenty determinations in a day. One lobe of the thyroid gland of a guinea pig contains ample iodine for chemical estimation. The other lobe can be used for histological study in order to check the assay by that method if desired.

### THE INVERSE RELATIONSHIP BETWEEN CALCIUM AND PHOSPHATE IN THE BLOOD\*

By FRANKLIN C. McLEAN AND MARIE A. HINRICHS

(From the Physiological Laboratory of the University of Chicago, Chicago)

Using the frog heart method for observation of  $\text{Ca}^{++}$  concentrations, we have studied, *in vivo* and *in vitro*, the time curves and end-points of the reaction which occurs when the concentration of phosphate in the blood is augmented. The velocity of the reaction, as manifested by a fall in  $\text{Ca}^{++}$  concentration, varies with the concentration of phosphate, equilibrium being reached *in vitro* in 24 hours or less. When equilibrium is attained, the product  $\text{Ca}^{++} \times$  total phosphate is approximately constant. When the reaction occurs in the organism, its end-product is quickly removed from the blood, resulting in the fall in serum calcium reported by others. These studies support the hypothesis, advanced by others, of the formation, in the plasma, under certain conditions, of a colloidal, non-diffusible calcium-phosphate complex, and suggest that the well known inverse relationship between calcium and phosphate depends upon the formation and removal from the blood of this complex, whenever the concentration of either is augmented.

A similar reaction occurs in casein solutions and in saline solutions, but unless the equilibrium product is greatly exceeded, is initiated only at pH 7.6 or higher, the velocity of the reaction being also greater with increasing alkalinity. Since the reaction is favored by alkalinity and since the precipitate formed in saline solutions is  $\text{Ca}_3(\text{PO}_4)_2$ , we incline to the belief that the complex formed in plasma is of similar composition. The fate of the complex in the organism is being investigated.

\* This work has been aided by a grant from the Josiah Macy, Jr., Foundation.

**THE SEPARATION OF A HISTAMINE-LIKE SUBSTANCE FROM  
HYDROLYZED PROTEINS**

By THOMAS L. McMEEKIN

*(From the Department of Physical Chemistry, Harvard Medical School,  
Boston)*

In separating the histidine fraction of hydrolyzed proteins a substance was obtained differing in certain respects from histidine. Unlike histidine it is precipitated by mercuric chloride in the presence of acetic acid and differs from histamine in that it is completely precipitated by mercuric sulfate in sulfuric acid solutions. A crystalline nitrate has been prepared from hydrolyzed gelatin and edestin respectively, suggesting that it is a constituent of both animal and vegetable proteins. The analytical data on the crystalline nitrate, though still inadequate to prove structure, suggest that the imidazole group is present, since the diazo reaction is positive.

The crystalline nitrate produces a marked fall in blood pressure when injected intravenously. Abel and Kubota in 1919 and Hanke and Koessler in 1920 reported blood pressure-reducing fractions of proteins, which may have contained the crystalline compound that has now been isolated. The fall in blood pressure produced by the crystalline nitrate, though qualitatively similar to that produced by histamine, differs in being less effective per unit of weight. Neither the preparative procedure nor the analytical data indicate histamine.

**IS CYSTINE SULFOXIDE AN INTERMEDIATE IN THE OXIDATIVE  
METABOLISM OF CYSTINE?**

By GRACE MEDES

*(From the Lankenau Hospital Research Institute, Philadelphia)*

The metabolism of cystine sulfoxide ( $C_6H_{12}O_6N_2S_2$ , synthesized by Toennies and Lavine) in the human body has been studied in comparison with that of cystine in respect to: (a) completeness of oxidation, as shown by the neutral sulfur in the urine and by the normal cystine output (method of Shinohara, unpublished); (b) rate of oxidation, as shown by the rate of excretion of sulfates; (c) ability to replace cystine in the diets of rats.

The evidence lends support to the theory that this compound may be an intermediate in the metabolism of cystine.

## ON THE SUGAR RADICALS OF SOME "MUCOIDS"

BY KARL MEYER AND JOHN W. PALMER

(From the Chemical Laboratory of the Department of Ophthalmology of the College of Physicians and Surgeons, Columbia University, New York)

Recently we reported<sup>22</sup> on the isolation of a sulfur-free nitrogenous polysaccharide acid from the vitreous humor of cattle. A search was made for similar substances from other sources.

By the same procedure a nitrogenous sulfur-free polysaccharide acid was obtained from human umbilical cord. The ease with which the sugar radicals from these two sources are isolated suggests that the mucins contain the sugar acids combined in a salt-like linkage with a basic protein.

In contrast to these, commercial gastric mucin gave a sulfur-free polysaccharide in stable combination with a polypeptide.

By this procedure cornea gave no sugar-containing material, but after treatment with weakly alkaline sulfide a sulfate-containing mucoid dissolved, which upon acidification yielded a compound resembling the mucoitinsulfuric acid of Levene and López-Suárez.<sup>23</sup> A similar product was obtained by alkaline hydrolysis.

The gonad-stimulating hormone from pregnancy urine was also investigated. Data on its amino sugar, reducing sugar, and nitrogen content indicate that this hormone should be classified with the "mucoids."

## SPECTROSCOPIC EVIDENCE FOR DIFFERENT MODIFICATIONS OF CHOLESTEROL RESULTING FROM SIMPLE CHEMICAL TREATMENT

BY E. S. MILLER, F. P. ZSCHEILE, ELIZABETH M. KOCH, T. R. HOGNESS, AND F. C. KOCH

(From the Departments of Chemistry and Physiological Chemistry, the University of Chicago, Chicago)

Koch and Koch demonstrated differences in the ability of cholesterol to acquire antirachitic potency by irradiation as a result of simple chemical treatment such as heating the cholesterol in acid or alkaline solutions. The recent work of Waddell showing the antirachitic potency of certain irradiated cholesterol preparations when fed to chicks supports these conclusions. From our

<sup>22</sup> Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **107**, 629 (1934).

<sup>23</sup> Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, **36**, 105 (1918).

previous knowledge of the absorption spectra of what we have regarded as pure cholesterol, such activation by irradiation is impossible, inasmuch as our pure cholesterol was transparent to ultra-violet light down to 2100 Å. It seems probable that different investigators are working with different forms of cholesterol. These discrepancies led us to undertake a thorough study of the change in cholesterol when subjected to simple chemical treatment. As evidence of these changes, we are using the absorption spectra as criteria.

The method used in obtaining absorption spectra is that involving a monochromator, a photoelectric cell, an electrometer tube, and hydrogen arc. The spectral range is from 2150 Å. to 7000 Å. with an accuracy in determining the absorption coefficient of less than 1 per cent error.

We have evidence that simple chemical treatments result in marked changes in the absorption spectrum of cholesterol, which can only be interpreted as resulting from different isomeric forms.

The bearing of this work upon known modifications of cholesterol is considered.

### PEPTIC HYDROLYSIS OF LACTALBUMIN

BY LILA MILLER

*(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)*

The type of linkage cleaved by the action of pepsin on proteins has been studied by comparing the liberation of amino and carboxyl groups. Sørensen and Waldschmidt-Leitz established a 1:1 ratio for casein, gelatin, ovalbumin, histone, and gliadin. In this study a 1:1 ratio has been found for lactalbumin. The methods used were the Van Slyke amino nitrogen method, the Willstätter, the Sørensen formaldehyde, and a modified Harris titration. The most rapid hydrolysis occurs during the first 4 hours, 10.4 per cent of the total nitrogen being liberated as amino nitrogen, whereas 14.9 and 18.6 per cent are liberated in 24 hours and 7 days, respectively.

The extent of hydrolysis at equilibrium depends upon the concentration of enzyme. With an enzyme to substrate ratio of 1:5,

the cleavage is equivalent to 24 per cent of the total nitrogen (Van Slyke). With ratios 2:5, 4:5, and 6:5, the cleavage is equivalent to 25, 26, and 31 per cent, respectively.

The acid concentration (pH 1.5 to 2) is not responsible for a significant portion of the hydrolysis. The amino nitrogen liberated in 7 days (30°) is 0.4 per cent of the total nitrogen. Partial enzymatic hydrolysis does not increase the susceptibility to acid hydrolysis, as is shown by maintaining heat-inactivated mixtures which have been digested to the extent of 7, 10, 11, 22, and 25 per cent at 30°. The changes are almost within the experimental error. Furthermore, heating the digestion mixtures to a temperature of 80–85° for 5 minutes does not cause further hydrolysis regardless of the extent of cleavage at the time.

#### SPECTROPHOTOMETRIC STUDIES ON THE LIEBERMANN-BURCHARD REACTION FOR STEROLS

By KENNETH MORGAREIDGE

*(From the Department of Biochemistry and the Institute of Optics, The University of Rochester, Rochester, New York)*

Quantitative spectrophotometry of the color produced in the Liebermann-Burchard reaction has been carried out for several sterols. The technique employed makes use of the Bausch and Lomb spectrophotometer, for which a special water-jacketed absorption cell was constructed. It was found that highly consistent results could be obtained only by means of rigid temperature control and the use of absolutely anhydrous chloroform.

Among the compounds studied were cholestene, allocholesterol, cholesterol, coprosterol, and cholestenone, which were found to give identical absorption spectra upon reaction with acetic anhydride and sulfuric acid. The absorption is confined to three bands, one in the violet below 4500 Å., one in the orange at 6200 Å., and one in the red at 6750 Å.

Variations in structure, limited to the presence or absence of a double bond or hydroxyl group, or the position of the double bond, in Rings A and B of the sterol nucleus, make a considerable difference in the rate of formation of the colored compound, as measured by the time necessary for maximum absorption at 6200



Å. to be reached. Thus, cholestene and cholestenone, with one and two double bonds, respectively, and no hydroxyls, required 5 and 300 minutes; cholesterol required 25 minutes, and allo-cholesterol and coprosterol 14 and 70 minutes, respectively.

### GLYCOLYSIS AND GLUTATHIONE

By SERGIUS MORGULIS

*(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha)*

Various bloods were studied from the point of view of the changes in the reduced glutathione (GSH) occurring during glycolysis. It was found to be generally the case that the GSH either increases or remains unchanged during the glycolytic process, but if glycolysis is suppressed or when the glycolytic process is far advanced or actually completed, the GSH diminishes to a greater or less extent. In hog bloods, those which do not glycolyze (about 60 per cent) show a decrease in the GSH of from 1.0 to 5.6 mg. per cent, whereas in the glycolyzing samples the GSH remains unchanged or, as was the case in a few bloods, an actual increase of over 2.0 mg. per cent occurs. Similar results were obtained with dog, rabbit, and human bloods. The behavior of goose blood is rather peculiar. The fresh blood, which practically does not glycolyze at all, shows a marked and continuous rise in the GSH during the incubation. Treated with KCN, the blood glycolyzes quite actively, and the GSH curve, though it resembles closely the GSH curve of the non-glycolyzing untreated blood, show a much smaller rise, especially after the 2nd hour of incubation. Experiments with rabbit blood show that added GSH has absolutely no effect on the progress of glycolysis and disappears very rapidly. Increasing the glycolytic activity of the blood with KCN does not influence the GSH curve, but suppressing the glycolysis with NaF brings about a loss in GSH which after the 6th hour becomes very rapid.

**SOME EFFECTS OF FATIGUE ON THE ACID-BASE BALANCE OF THE  
BLOOD SERUM OF THE NORMAL DOG**

BY MINERVA MORSE AND FREDERIC W. SCHLUTZ

*(From the Department of Pediatrics of the University of Chicago, Chicago)*

A study has been made of the changes in the acid-base balance of the blood serum of the normal dog as a result of exercise to exhaustion.

With exhaustion produced by swimming at 38° there was always a fall in protein concentration, accounted for by the drawing of blood samples and by dilution from the intake of water during the exercise. In all treadmill experiments (22 per cent incline) without water, increased protein concentrations indicated a concentration of blood with exercise. In treadmill experiments with water intake, there was little change in protein concentration. The total salt concentrations changed in a similar manner and were accounted for almost entirely by dilution or concentration of the blood. However, such an explanation did not suffice for the serum bicarbonate, lactate, and phosphate changes.

A decrease in serum bicarbonate with swimming was with few exceptions quantitatively accounted for by entrance of lactic acid into the blood; with treadmill exercise the decrease in bicarbonate was often greater than the increase in lactate, but in such cases there was a compensatory fall of base or rise in chloride. With one exception phosphate concentrations decreased, regardless of the mode of exercise.

The total fixed base concentrations found agreed within the limits of experimental error with those calculated by adding together the anions determined (bicarbonate, chloride, lactate, phosphate, and proteinate), both before exercise and at exhaustion; this is evidence that no other anion entered or increased in the blood during exercise in quantities detectable by our methods.

**UREA CLEARANCE DURING NORMAL PREGNANCY**

BY MARGARET NICE

*(From the Laboratory of Maternity Hospital, Western Reserve University,  
Cleveland)*

The changes in urea clearance which have been reported for normal and toxemic pregnancy indicate that pregnancy may

have a fundamental effect upon kidney function. This is further emphasized by the characteristic low blood urea of pregnancy, which could result from an increased kidney function.

To establish the urea clearance rate during normal pregnancy, 95 tests were run in series on thirteen normal women from the 5th month ante partum to the 8th month post partum. Clearances were also run on normal non-pregnant women, and before and after feeding urea to pregnant women.

The results show that there is no significant variation of the urea clearance rate from month to month during pregnancy, but that the ante partum mean of 157 per cent is significantly higher than the post partum mean of 96 per cent, or than that for the non-pregnant normals, of 105 per cent. The last two figures are not significantly different, and closely approximate Van Slyke's normal of 100 per cent. The feeding of urea had no effect upon the elevated clearance rate of pregnancy. This establishes a high urea clearance rate, and hence an increased kidney function, during normal pregnancy. It may in turn be responsible for the low blood urea.

Although kidney function has been studied chiefly from the point of view of explaining kidney deficiency, the mechanism of this increased urea clearance rate can be discussed by using the conclusions of those findings which may logically be reversed.

#### THE IONIZATION OF LACTIC ACID FROM ZERO TO FIFTY DEGREES

By LESLIE FREDERICK NIMS AND PAUL K. SMITH

*(From the Departments of Physiology, and of Pharmacology and Toxicology,  
Yale University School of Medicine, New Haven)*

The thermodynamic ionization constant of lactic acid has been determined at 12.5° intervals from 0–50°. Electromotive force measurements were made of cells without liquid junction containing buffer solutions of hydrochloric acid with lithium, barium, or strontium lactate.

The measurements indicate that lactic acid has a maximum ionization at 23.6°, pK 3.866 at 25°, and pK 3.877 at 37.5°. The free energy and heat of ionization are given as functions of the temperature.

**WATER DISTRIBUTION IN THE BLOOD OF PREGNANT AND NON-PREGNANT WOMEN**

BY FRED W. OBERST AND E. D. PLASS

*(From the Department of Obstetrics and Gynecology, State University of Iowa, Iowa City)*

The distribution of water between plasma and cells was studied in 60 women: including ten non-pregnant, twenty late in pregnancy, ten early in labor, ten at delivery, and ten during the puerperium (7 to 9 days after delivery). Umbilical cord blood was drawn immediately after birth. Determinations included the specific gravity and water content of whole blood and plasma, the cell volume and hemoglobin of whole blood, and the total proteins of plasma. The data on the red blood cells were calculated.

The higher water content of plasma and red cells in pregnant than in non-pregnant women was associated with decreased specific gravity and diminished total plasma protein. The cell volume and hemoglobin concentration of whole blood were decreased in the pregnant women, but the hemoglobin concentration in the cells was increased.

Early in labor the plasma and cells became slightly more concentrated. The water content was decreased, while the specific gravity, plasma protein, cell volume, and hemoglobin were increased. At the time of delivery, these changes were considerably more marked.

The water content of plasma and cells in cord blood was higher than in maternal blood. Even though the cell volume and hemoglobin of fetal whole blood were much higher than in maternal blood, the hemoglobin per kilo of water was lower.

After delivery, the average values for the various constituents approached those of the non-pregnant group, although the hematocrit and hemoglobin remained higher and the water content of both plasma and cells was lower.

THE EFFECT OF UNDERNUTRITION AND OF SPECIFIC VITAMIN  
DEFICIENCY ON THE LIVER LIPIDS OF CHOLESTEROL-  
FED RATS

BY RUTH OKEY AND HELEN L. GILLUM

(From the Laboratory of Household Science, University of California,  
Berkeley)

The general plan used for feeding the rats and the technique for analysis of tissues have been described in previous papers from this laboratory. Litter mates, paired as to weight and sex, were placed at weaning on the experimental diets, and, as a rule, half of each litter was given cholesterol to the extent of 1 per cent of the total food eaten and half fed the corresponding diet without it.

The "undernourished" animals were limited to the same weight gain as the vitamin A-deficient animals. The rats were killed at approximately 100 days of age and the moisture content, fatty acids, total and free cholesterol, and lecithin of the livers determined. In the *underfed* rats the percentage of liver cholesterol ester was higher and the total fatty acid somewhat lower than in the cholesterol-fed controls. The vitamin A-deficient animals had almost as high a percentage and approximately three-fourths as great a total amount of liver cholesterol as their controls.

This was in marked contrast to the cholesterol-fed animals deprived of a source of *vitamin B<sub>1</sub>* 30 days before killing, in that the latter had average liver cholesterol values of less than 1 per cent in contrast to control values of 4 to 8 per cent. Neutral fat was very low in this group but was not affected to a significant extent.

The *vitamin G-deficient* cholesterol-fed animals had liver cholesterol values varying from 1.5 to 4 per cent, with total fatty acids averaging about 11 per cent.

THE ABSORPTION SPECTRUM AND OTHER PROPERTIES OF  
VITAMIN E

BY H. S. OLCOTT

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

A band at 2940 Å. has previously been detected in the ultra-violet absorption spectra of vitamin E concentrates from wheat germ and cottonseed oils. The presence of this band in the spectrum of material prepared in an exactly similar manner from palm

oil but devoid of vitamin E activity is further evidence that the band is the property of some other constituent of wheat germ and cottonseed oils, and not a property of the vitamin. Vitamin E is destroyed by methylation with dimethyl sulfate or with methyl iodide and silver oxide. Hydrogenation over Ni at 250 to 280 atmospheres and 230° suffices neither to saturate the concentrate nor to destroy the vitamin activity.<sup>24</sup> These and previous results suggest that vitamin E contains a hydroxyl group and a double bond. The double bond adds halogens easily and hydrogen with difficulty if at all.

#### ESTIMATION OF AMINO SUGAR

By JOHN W. PALMER AND KARL MEYER

*(From the Chemical Laboratory of the Department of Ophthalmology of the College of Physicians and Surgeons, Columbia University, New York)*

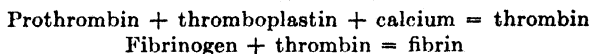
The colorimetric amino sugar method of Elson and Morgan,<sup>25</sup> based on the reaction of Ehrlich's reagent with the condensation product of acetylacetone and amino sugar, does not follow Beer's law over the range originally suggested. Furthermore, with hydrolysates of some sugar-containing complexes the colors obtained do not match those with the glucosamine hydrochloride standards. These errors were eliminated by spectrophotometric comparison when samples containing 0.05 to 0.3 mg. of glucosamine hydrochloride were used.

#### THE PROTHROMBIN IN HEMOPHILIA AND IN OBSTRUCTIVE JAUNDICE

By ARMAND J. QUICK

*(From the Department of Surgery of the Fifth Avenue Hospital, New York)*

On the assumption that blood clotting proceeds in two steps:



and that the rate of clotting is proportional to the concentration of thrombin, a means for the determination of prothrombin is

<sup>24</sup> I am indebted to Dr. Homer Adkins of the University of Wisconsin for the hydrogenation.

<sup>25</sup> Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933).

proposed. If the first phase proceeds according to the law of mass action, the rate of thrombin formation is a product of the concentration of prothrombin, thromboplastin, and calcium. When oxalated plasma is used and recalcified with the optimal amount of calcium (0.1 cc. of 0.025 M calcium chloride added to 0.1 cc. of plasma obtained by mixing 9 cc. of blood with 1 cc. of 0.1 M sodium oxalate), and an excess of thromboplastin added (obtained from rabbit brains), only prothrombin is left as a variable and its concentration should determine the clotting time. Oxalated plasmas of man, dog, and rabbit on recalcification clot approximately in 120 seconds, but with excess thromboplastin, human plasma clots consistently in 22 to 25 seconds, dog plasma in 10 seconds, and rabbit plasma in 12 seconds, which indicates a much higher concentration of prothrombin in dog and rabbit blood than in that of man. Plasma from five cases of hemophilia clotted as rapidly as the normal, *i.e.* 22 to 25 seconds, with excess of thromboplastin. This suggests that in hemophilia the prothrombin is normal in quantity and quality, but thromboplastin is deficient. Plasma from various types of obstructive jaundice often showed delayed clotting, as long as 90 seconds in the presence of excess thromboplastin. Thus, the hemorrhagic diathesis of obstructive jaundice appears to be due to a deficiency of prothrombin.

#### STUDIES ON THE ACID-BASE CONDITION OF BLOOD

##### V. THE INFLUENCE OF PROTEIN CONCENTRATION ON THE COLORIMETRIC pH DETERMINATION OF BLOOD SERUM

BY HOWARD W. ROBINSON, J. WAIDE PRICE, AND  
GLENN E. CULLEN

*(From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)*

We have studied further the effect of increase and decrease of protein concentration on the *C* correction at approximately constant CO<sub>2</sub> tension, total CO<sub>2</sub>, and pH by concentrating dog sera in an ultrafiltration apparatus. For the dilution experiments the original serum was diluted with its ultrafiltrate. The samples were equilibrated in the Simms electrode and the CO<sub>2</sub> and pH determinations made as previously described.

We have found, with twenty-five sera, a relationship similar to that reported by Myers *et al.* for every individual sample of serum. The change of *C* correction per gm. of protein between 4 and 6.5 gm. of protein per 100 cc. is about 0.02 pH unit. However, the variations in the *C* corrections in samples of sera taken from the same animal at different times have no consistent relationship to the protein concentration. Thus, for example, in Dog F the *C* values for seven sera, taken at different times, varied between 0.28 and 0.39, while the protein concentration varied only between 5.8 and 7.3 per cent. Even with this slight change in protein there was no association between high and low protein and high and low *C* values.

From these experiments we conclude that although there is a definite relationship between the protein concentration and the *C* value, there must be other factors which have greater influence than change in protein concentration.

**A COLORIMETRIC METHOD FOR THE DETERMINATION OF ASCORBIC ACID AND A COMPARISON OF RESULTS OBTAINED BY THIS PROCEDURE AND THE DICHLOROPHENOL INDOPHENOL TITRATION METHOD**

By JOSEPH H. ROE

(From the Department of Biochemistry, School of Medicine, George Washington University, Washington)

A colorimetric method has been developed for the determination of ascorbic acid which is based upon the formation of furfural from ascorbic acid when the latter is boiled with HCl and the development of a color by the reaction of aniline with the furfural. Acetic acid is used as a buffer against excess aniline and the color is stabilized by the use of an antioxidant, SnCl<sub>2</sub>. Ascorbic acid exists in foods in a reduced form and a reversibly oxidized form. Only the reduced form of ascorbic acid gives furfural under the conditions of this procedure, but the oxidized form may be readily determined by boiling with HCl containing SnCl<sub>2</sub>. A comparison of the ascorbic acid content of foods determined by this method and Tillmans' 2,6-dichlorophenol indophenol titration procedure has been made. Good agreement between the two methods was obtained upon fresh foods, but foods that have aged show a higher value by the colorimetric method because Tillmans' method does



not measure the reversibly oxidized ascorbic acid. A study of the antiscorbutic value of reversibly oxidized ascorbic acid is being made.

#### DETERMINATION OF INDICAN IN URINE

BY ANTON R. ROSE AND WILLIAM G. EXTON

WITH THE ASSISTANCE OF FRED SCHATNER, MARY MCCARTHY, AND  
E. HORNING

*(From the Laboratory and Longevity Service of The Prudential Insurance  
Company of America, Newark)*

The formation of indigo from indican by iron chloride in concentrated HCl is prevented by resorcinol, which gives a red substance with indoxyl. This red color is more intense than the indigo blue of the Obermayer or Jolles methods, and the difference in intensity becomes even greater when extracted with amyl acetate. Some resorcinol derivatives are better for this purpose than resorcinol; the side chains which give the best results are primary and secondary hexyl, dibutyl, and octyl.

Cupric bromide is a more efficient oxidizing agent than iron chloride; 1 drop of the resorcinol derivative in 1/20 saturated alcoholic solution is added to 1 cc. of the urine sample, which is then mixed with 2 cc. of 0.5 per cent solution of cupric bromide in concentrated HCl. After standing a minute or two, the red compound is then extracted with 4 cc. of amyl acetate.

Since blocking of the indoxyl molecules by the dihydroxybenzene may not be complete, with resultant bluish admixture in the cherry, the color comparison is preferably made in a scopometer in which measurements of color density are not disturbed by slight color admixtures or shifts of hue. Since, even when obtainable, indican does not keep well, we calibrate the scopometer with solutions of pure indigo and construct a calibration curve for the indican test by referring the indican to the indigo curve. This is done with samples of urine rich in indican from which indigo is obtained and estimated quantitatively by permanganate titration.

ISOLATION OF THE "UNKNOWN ESSENTIAL" PRESENT IN  
PROTEINSBY WILLIAM C. ROSE, RICHARD H. MCCOY, CURTIS E. MEYER,  
HERBERT E. CARTER, MADELYN WOMACK, AND  
EDWIN T. MERTZ*(From the Laboratory of Physiological Chemistry, University of Illinois,  
Urbana)*

Investigations in this laboratory during the current year have demonstrated the fact that our so called "unknown essential" consists of two factors,<sup>26</sup> which until recently have been designated as Unknown I and Unknown II. Of these, the former is much more soluble in butyl alcohol than is the latter. Unknown I was promptly identified as isoleucine. This amino acid was not present in sufficient amounts in our former basal diet. This fact, and the reasons therefor, will be discussed later.

The discovery that isoleucine was one of the limiting factors greatly expedited the separation of the other compound. The latter (Unknown II) has now been obtained in pure, crystalline form. The essential features in its separation are as follows: removal of the less soluble amino acids from a hydrolyzed protein by direct crystallization, preparation of the copper salts of the remaining materials, and exclusion of those which are relatively insoluble in water. The water-soluble copper salts are then freed of copper, and the aqueous solution is repeatedly extracted with large volumes of butyl alcohol. The combined solid material recovered from Extracts 4 to 17 inclusive is then redissolved in water and reextracted a few times with butyl alcohol for the removal of the remainder of the isoleucine. The water layer is treated with phosphotungstic acid under appropriate conditions, and the filtrate is subjected to fractional crystallizations with different concentrations of alcohol.

The pure product has a uniform crystal structure, and promotes excellent growth when included in the basal diet to the extent of 0.4 to 0.5 per cent. Analyses of it and its derivatives, together with certain other properties, show that it is one of the  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids. On reduction it yields  $\alpha$ -amino-*n*-butyric acid. Its spatial configuration, as demonstrated by synthesis, is identical with one of the two stereoisomeric  $\alpha$ -amino- $\beta$ -

<sup>26</sup> Womack, M., and Rose, W. C., unpublished data.

hydroxy-*n*-butyric acids obtained from isocrotonic acid. These will be resolved and compared chemically and physiologically with the natural product in the near future.

**STUDIES ON GROWTH FACTORS; THEIR EFFECT ON THE  
GROWTH OF CERTAIN PATHOGENS, AND THEIR EF-  
FECT ON *ESCHERICHIA COLI*\***

By MELVILLE SAHYUN

(From the Department of Bacteriology and Experimental Pathology, Stanford University, California)

The sources of growth factors investigated were derived from peptone and red blood corpuscles. Two procedures were employed: (a) extraction by alcohol, such as ethyl and butyl alcohol, precipitation with phosphotungstic acid, and ultrafiltration; (b) acid hydrolysis and repeated extraction of the hydrolysate by ethyl alcohol.

By extraction with alcohol two different growth factors were observed: (1) a butyl alcohol-soluble and (2) a butyl alcohol-insoluble factor. Both were ultrafiltrable through cellophane No. 600, showing a molecular weight approximating that of a disaccharide. They behaved like organic acids.

By acid hydrolysis the growth factor prepared appeared to be similar to the non-butyl alcohol-soluble factor. The butyl alcohol-soluble factor is destroyed by acid hydrolysis.

*Effect on Pathogens*—Pathogens grew luxuriantly upon repeated reculturing for over a month in a buffered synthetic medium containing essential amino acids and the butyl alcohol-insoluble growth factor. With one exception, in the same synthetic medium without this growth factor, the same microorganisms did not survive repeated reculturing.

*Effect on Escherichia coli*—In a synthetic medium containing nitrogen as ammonium sulfate, *Escherichia coli* grew very slowly. The addition of the growth factor in minute amounts accelerated the rate of growth and carbohydrate metabolism of *Escherichia coli*. *Escherichia coli* was also observed to secrete a growth factor, the properties of which resembled those of the butyl alcohol-insoluble factor. This factor was adsorbed on activated charcoal and recovered.

\* Supported in part by a grant from Eli Lilly and Company, Indianapolis.

**MINERAL PARTITION DURING INTESTINAL DIGESTION**

BY PHILIP J. SCHAIBLE, S. L. BANDEMER, AND J. M. MOORE

*(From the Chemical Laboratory, Michigan Agricultural Experiment Station,  
East Lansing)*

While there is considerable doubt as to all the factors involved in absorption from the intestine, most theories specify intimate contact of the substance in solution with the surface of the tract. Thus, since it is the liquid phase of the intestinal contents that is important for absorption at any point, the analysis and reaction of this medium are of particular interest.

Rations of normal and high mineral contents were fed to chicks for 5 weeks. Blood and the right tibia were obtained for analysis and the gastrointestinal tract removed. The small intestine was divided into three approximately equal sections and their contents expressed. In order to obtain the liquid phase from small quantities of intestinal contents, a portion of the material was blotted with filter paper. The absorbed liquid thus obtained and an aliquot of the total contents were analyzed for mineral constituents.

With the data obtained in this manner, an attempt was made to determine the influence of normal and high mineral rations on the composition of the liquid and total contents of the intestine. It was found that the concentration of mineral in the absorbable liquid phase was not a reflection of that in the ration, as one might reason. As a result, mineral ratios were frequently very different from those considered optimum in the diet. There were indications that the organic as well as the inorganic part of the ration influenced the concentrations found.

**SPECIFICITY OF STEROL ABSORPTION**BY RUDOLF SCHOENHEIMER, WARREN M. SPERRY, AND  
HENRIK DAM*(From the Department of Biological Chemistry, College of Physicians and  
Surgeons, Columbia University, New York)*

It has been shown previously that the absorption of sterols from the intestinal tract is highly specific in regard to chemical structure. While cholesterol is easily absorbable, other sterols, such as phytosterols and coprosterol, with small differences in chemical structure, are practically non-absorbable.

The sterol most similar to cholesterol is allocholesterol, which differs only by a shift of the double bond from  $\Delta 5$  to  $6$  to  $\Delta 4$  to  $5$ . It has been found in experiments on mice and dogs that this small change in structure is sufficient to interfere markedly with absorbability.

Ostreasterol, isolated from oysters, and kindly given to us by Dr. Werner Bergmann of Yale University, was absorbed only to a slight degree by mice. This sterol is of particular interest, since it is of animal origin but closely related in structure to the plant sterol, sitosterol.

Some experiments concerning the specificity of sterol esterases and their relation to sterol absorption will be discussed.

#### THE AVAILABILITY OF TISSUE NUTRIENTS AFTER ALCOHOL EXTRACTION AND AFTER HEATING\*

By WALTER H. SEEGERs

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

The impaired nutritive value of liver and other animal tissues as the result of alcohol extraction or of heating has been explained as due either to the removal of a necessary dietary constituent or to damage done to the protein. Since liver is the most vulnerable among the animal tissues, it has been studied in greater detail. An otherwise adequate ration containing alcohol-extracted liver (130 hours) as the source of protein at a 15 per cent level does not support growth in rats. This is due to an impaired digestibility and a decrease in the biological value of the protein as well as to the removal of valuable nutrients. When this extracted liver is hydrolyzed with  $H_2SO_4$  before incorporation in the ration with tryptophane, growth is more rapid than on the unhydrolyzed preparations.

Heating dried whole liver for 2 weeks at  $100^\circ$  lowers its biological value only slightly, but also decreases its digestibility. Heating at  $120^\circ$  for 3 days lowers the digestibility to that of 130 hours alcohol-extracted liver. Confirmatory *in vitro* digestion experiments also included a study of beef heart, round, and kidney. The protein of heated liver is also made more available for metabolic

\* Supported in part by a grant from the National Live Stock and Meat Board through the Committee on Grants of the National Research Council.

purposes by acid hydrolysis. The impairment of the nutritive value of animal tissue proteins by alcohol extraction and by heating at 100–120° seems to be largely a decrease in their digestibility; the amino acids themselves are not altered.

### THE GASOMETRIC DETERMINATION OF CHLORIDE IN SERUM AND URINE

By JULIUS SENDROY, JR.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York)*

A rapid, accurate chloride method has been developed, with 1.0 or 0.5 cc. of serum, based on the principle of the differential solubility of difficultly soluble salts having a common ion.

The procedure, in general, is as follows: The material to be analyzed is diluted with 0.5 per cent  $\text{H}_3\text{PO}_4$ , so that the chloride content is about 10 mm per liter. Without further treatment, solid  $\text{AgIO}_3$  is added to the solution, and the whole shaken vigorously for 1 minute. The reaction, which is almost instantaneous, is  $\text{AgIO}_3 + \text{NaCl} \rightarrow \downarrow \text{AgCl} + \text{NaIO}_3$ . The silver chloride is filtered off or centrifuged, and the iodate in solution is then measured manometrically, according to the reaction  $3\text{N}_2\text{H}_4 + 2\text{NaIO}_3 \rightarrow \uparrow 3\text{N}_2 + 2\text{NaI} + 6\text{H}_2\text{O}$ . The iodate may also be measured titrimetrically, and in some cases, colorimetrically, by the addition of KI.

The removal of proteins from serum or urine is unnecessary.

For all measurements, suitable blanks are performed.

The accuracy of the macromethod outlined above is 0.5 per cent. A micromethod, with 0.2 or 0.1 cc. of serum, is now being developed.

### TITRATION OF MOUSE TUMORS

By M. J. SHEAR

*(From the Office of Cancer Investigations, United States Public Health  
Service, Harvard Medical School, Boston)*

In therapeutic experiments positive results may be overlooked if the effect of the treatment on tumor growth is obscured by uncontrolled biological variations. Variations in the reactions of the animals were minimized by the employment of mice of genetically

pure strain. Immunological reactions were minimized by the employment, for transplanting, of tumors which had arisen originally in mice of the same pure strain. Variations in the growth properties of the different portions of the tumors used in transplanting were avoided by the use of equal volumes of homogeneous tumor suspensions.

Healthy tumor tissue, after mincing, was shaken with balanced salt solutions containing gelatin. The latter served the double purpose of retarding sedimentation and of counteracting the destructive swelling of tumor cells which occurs in protein-free solutions. After light centrifuging, equal volumes of the homogeneous suspensions were used for the inoculation of other mice.

With concentrated suspensions, takes were obtained in all cases. Highly diluted suspensions gave rise to no tumors. Intermediate dilutions were sought which gave rise to tumors in about 50 per cent of the cases. With different tumors, this mid-point in the titration occurred at different dilutions. Two strains of mice were employed, and titrations were carried out with two tumors of each strain. Duplicate titrations gave good agreement.

This more quantitative technique is now being employed in the therapeutic experiments. When dilute suspensions are used, the time required for the development of the tumors is materially lengthened, thus increasing the period available for assays of preventive and curative treatments.

#### **EFFECTS OF NITROGENOUS SUBSTANCES UPON SUGAR DETERMINATION**

BY FAY SHEPPARD AND MARK R. EVERETT

*(From the Department of Biochemistry and Pharmacology, University of Oklahoma Medical School, Oklahoma City)*

By quantitative studies of nitrogenous compounds we have determined Sumner and Folin-Wu glucose equivalents, effects upon simultaneous oxidation of glucose, and permissible concentrations in analytical samples. Nitrogenous phenols, diphenols, creatine, creatinine, and bile pigments reduce Sumner's reagent slightly. These substances and diamines, sulphydryl derivatives, cyclic amino acids, hydantoins, and purines reduce the Folin-Wu reagents. Sumner to Folin-Wu ratios for these substances are low,

only creatine and bile pigments exceeding 0.10. Higher ratios indicate the presence of carbohydrates.

Diphenols, especially dihydroxyphenylalanine, hydroquinone, and epinephrine, are powerful reductants whose Folin-Wu equivalents resemble those of monosaccharides. In appropriate concentrations they catalyze the oxidation of glucose, epinephrine being most active. Glucose oxidation by Sumner's reagent is decreased by many amino acids, increased by some; by the Folin-Wu reagents, increased by amino acids, hydantoins, etc., decreased by larger amounts of amino acids, by ammonia, cyanates, nitrites, thiocyanates, tyramine, and xanthine. Here cystine reduces as powerfully as cysteine. Thiocyanates bleach reduced phosphomolybdic acid completely. Glucose oxidation by Sumner's reagent is depressed by amines and amino acids in proportion to their amino nitrogen.

Experiments with artificial urine demonstrate that Sumner's method gives low values for urine sugar, the Folin-Wu method high values. Acid hydrolysis produces nitrogenous material which reduces only the copper reagent; therefore hydrolyzable sugar is more correctly determined by Sumner's method. This substance is removed by Lloyd's reagent. The latter also removes one-third of ammonia and glucosamine. The only reducing substances completely removed are bile pigments, creatinine, diiodotyrosine, and uric acid.

#### DETERMINATION AND METABOLISM OF CITRIC ACID

By CAROLINE C. SHERMAN, LAFAYETTE B. MENDEL, AND  
ARTHUR H. SMITH

(From the Department of Physiological Chemistry, Yale University,  
New Haven)

The Pucher, Vickery, and Leavenworth method for the determination of citric acid has been adapted to a stufenphotometric estimation of 0.01 to 1.50 mg. quantities of citric acid. The method has been applied satisfactorily to the analysis of blood, urine, feces, tissues, and other biological materials. Simultaneous blood and urine studies have been made in animals to which citric acid has been administered *per os*.

The effect of a variety of factors on the citric acid content of



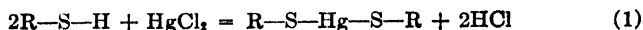
blood and urine in dogs on a synthetic, "citrate-low" diet has been determined.

**A PRECISION METHOD FOR THE DETERMINATION OF CYSTEINE  
APPLICABLE TO THE STANDARDIZATION OF  
CYSTEINE HYDROCHLORIDE**

BY KAMENOSUKE SHINOHARA

(From the Lankenau Hospital Research Institute, Philadelphia)

HgCl<sub>2</sub> combines rapidly and without forming a precipitate with cysteine in an acetate buffer of pH 5 in accordance with Equation 1



The color produced by phospho-18-tungstic acid and cysteine is in direct proportion to the concentration of cysteine, and independent of that of the former, provided it is present in excess.

Consideration of these two facts will give Equation 2, which is valid as long as the HgCl<sub>2</sub> is less than the equivalent amount of cysteine:

$$I = I_0 - (100/(100 - \alpha)) x \quad (2)$$

where I is the color intensity developed by the complex acid in a cysteine solution containing  $x$  N HgCl<sub>2</sub>,  $I_0$  that of a solution containing no HgCl<sub>2</sub> but the same amount of cysteine, and  $\alpha$  the percentage of impurities.

Therefore, the color intensities of solutions containing a definite amount of cysteine and varying amounts of HgCl<sub>2</sub> are measured. The amount of HgCl<sub>2</sub> just sufficient to combine with the cysteine is determined by actual experimentation, by graphical interpolation, or by the method of least squares, and the purity of the sample calculated. The color reaction is sensitive, the probable experimental error being  $\pm 0.2$  per cent. Thioglycolic acid can also be standardized by this method.

The water content of the sample can easily be determined by drying it on phosphorus pentoxide at room temperature. At 100° HCl is lost and cysteine decomposes. HCl determination, which is carried out easily, also aids standardization. Cysteine is determined by the sulfite and phospho-18-tungstic acid method in the aforesaid buffer.

Commercial cysteine hydrochlorides were found to be 82 to 94

per cent pure, the impurities being mainly water and a little cystine.

### RICKETS AND TETANY DUE TO LOW CALCIUM DIETS

By ALFRED T. SHOHL

*(From the Infants' and Children's Hospitals, and the Department of Pediatrics, Harvard Medical School, Boston)*

The ricketogenic properties of diets for rats have been investigated, especially those low in calcium and high in phosphorus.

The basal diet is that of Steenbock and Black, Ration 2965,<sup>27</sup> altered by various additions of  $\text{CaCO}_3$  and  $\text{KH}_2\text{PO}_4$ . The criteria of the effects produced have been histological examination, Roentgenograms, and ash analyses of the bones; quantitative determination of the serum calcium, phosphate, and protein; galvanic electrical reactions. The results show that the levels or absolute amounts of the calcium and phosphorus are as important as the ratios of these constituents. Provided both the levels and ratios are considered, not one given diet but a number of diets are thus offered in construction of rickets-producing diets. Rickets due to low calcium is not the mirror image of rickets due to low phosphorus, for when the proportion of phosphorus is too great, growth and bone growth are inhibited, and osteoporosis and not rickets results. High phosphorus-low calcium diets are associated with tetany and with lowered calcium and raised phosphorus in the serum. Between low calcium and low phosphorus diets lies a third group of ricketogenic diets, in which both calcium and phosphorus are low though neither is in excess of the other, and are in a proportion which has heretofore been considered as normal or optimal.

### THE PASSAGE OF FED ELAIDIC ACID INTO TISSUE PHOSPHOLIPIDS

By ROBERT GORDON SINCLAIR

*(From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)*

In order to test further the question of whether or not the phospholipids are intermediary products in the metabolism of the fatty

<sup>27</sup> Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**; 263 (1925).

acids, elaidic and erucic acids were fed. Since both of these acids are unsaturated and yet form lead salts which have a very limited solubility in cold alcohol, it was to be expected that their entrance into the phospholipids would be evident in an increase in the iodine number of the solid acid fraction of the phospholipid fatty acids. The available material being limited, a microprocedure was developed which permits the determination of the percentage and iodine number of the solid and liquid fractions on 20 to 30 mg. of fatty acids.

It has been found that elaidic acid can readily be detected in the tissue phospholipids and can comprise a fairly high percentage of the total phospholipid fatty acids. There is reason to believe that erucic acid also enters into the phospholipid molecule, but the evidence is much less convincing.

However, before this finding can be taken as evidence that the phospholipids are intermediary metabolites, engaged in the transport or combustion of the fatty acids, one outstanding premise must be fulfilled; *viz.*, the passage of elaidic acid into and out of the phospholipids must occur promptly on its addition to and removal from the diet. Experiments to test this premise are in progress.

#### CHOLESTEROL ESTERASE IN BLOOD

By WARREN M. SPERRY AND RUDOLF SCHOENHEIMER

*(From the Chemical Laboratory, Babies Hospital, and the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York)*

An enzymatic synthesis of cholesterol esters has been demonstrated in blood serum. Sera from normal human subjects were incubated at 38–40° in sealed tubes under sterile conditions. A marked decrease in free cholesterol with an equivalent increase in combined cholesterol was observed in every case. In twenty-three such experiments the average decrease in free cholesterol was 57.2 per cent of the original amount. Whereas in normal individuals the ratio of combined cholesterol to free cholesterol is always between 2.3 and 3.1 (equivalent to 70 to 75 per cent of combined cholesterol in total cholesterol), after incubation a minimum ratio of 4.0 and a maximum of 24.9 were found. A shift of fatty acids from other compounds to cholesterol seems to have

occurred. Synthesis of cholesterol esters in biological material *in vitro* does not appear to have been observed previously.

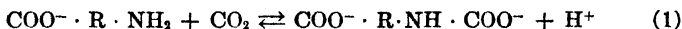
When hemolyzed whole blood was incubated under the same conditions, only slight esterification occurred, while with hemolyzed blood cells there was no change at all. Apparently a factor which inhibits cholesterol ester synthesis is present in erythrocytes. This may account for the fact that these cells contain practically no cholesterol esters.

**THE CARBAMATE-CARBON DIOXIDE EQUILIBRIUM OF AMINO ACIDS, HEMOGLOBIN, AND SERUM PROTEINS AND ITS SIGNIFICANCE IN THE CARBON DIOXIDE TRANSPORT OF THE BLOOD**

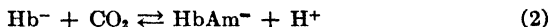
BY WILLIAM C. STADIE AND HELEN O'BRIEN

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia)

In the case of amino acids, the amphanion,  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_2$ , and not the zwitter ion,  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_3^+$ , reacts with  $\text{CO}_2$  to form carbamate



In the case of hemoglobin the analogous amphanion,  $\text{Hb}^-$  (*i.e.* BHb) and not the zwitter ion (uncharged protein) reacts to form hemoglobin carbamate.



The resultant carbamates of amino acids or hemoglobin are dibasic salts at  $\text{pH} > \text{about } 7$ .

The mass action equation for the equilibrium of amino acids and  $\text{CO}_2$  is

$$\frac{(\text{Am}^-) (\text{H}^+)}{(\text{COO}^- \cdot \text{R} \cdot \text{NH}_2)} = \alpha_{\text{CO}_2} K_{\text{Am}} P_{\text{CO}_2} \quad (3)$$

while for hemoglobin it is

$$\frac{(\text{HbAm}^-) (\text{H}^+)}{(\text{Hb}^-)} = \alpha_{\text{CO}_2} K_{\text{Am}} P_{\text{CO}_2} \quad (4)$$

Two cases arise.

1. Carbamate equilibrium when  $\text{H}_2\text{CO}_3$  formation is excluded. Carbamate formation is rapid, while  $\text{H}_2\text{CO}_3$  formation (particularly at low temperatures) is slow. Therefore by rapid equilibration and analysis the equilibrium between  $\text{CO}_2$  and amino acids or hemoglobin in the presence of varying quantities of base (pH initially 7.4 to 11) can be established and measured in the virtual absence of  $\text{H}_2\text{CO}_3$  or its ions  $\text{HCO}_3^-$  and  $\text{CO}_3^{--}$ . The total base (milli-equivalents per liter) is then respectively

$$(\text{B}^+) = (\text{COO}^- \cdot \text{R} \cdot \text{NH}_2) + 2(\text{Am}^-) \quad (5)$$

or

$$(\text{B}^+) = (\text{Hb}^-) + 2(\text{HbAm}^-) \quad (6)$$

Equations 3 and 5 or 4 and 6 may be combined to give an equilibrium equation showing the concentration of carbamate in terms of  $P_{\text{CO}_2}$  or  $\text{pH}^+$ . The respective values of  $K_{\text{Am}}$ , the mass action constant for the *amino acid-carbamate- $\text{CO}_2$*  equilibria, can be calculated from data on various amino acids.

2. Carbamate equilibrium when  $\text{H}_2\text{CO}_3$  formation is included. If sufficient time is allowed, the equilibrium also includes  $\text{H}_2\text{CO}_3$  and its ions  $\text{HCO}_3^-$  and  $\text{CO}_3^{--}$ . This equilibrium, which corresponds to the one in blood, gives quite different carbamate, pH, or  $P_{\text{CO}_2}$  relations from case (1). The base now is

$$(\text{B}^+) = (\text{COO}^- \cdot \text{R} \cdot \text{NH}_2) + 2(\text{Am}^-) + (\text{HCO}_3^-) + 2(\text{CO}_3^{--}) \quad (7)$$

$$(\text{B}^+) = (\text{Hb}^-) + 2(\text{HbAm}^-) + (\text{HCO}_3^-) + 2(\text{CO}_3^{--}) \quad (8)$$

As before, Equations 3 and 7 or 4 and 8 may be combined to give the mass action equation for the *amino acid-carbamate-carbonate- $\text{CO}_2$*  equilibria.

For amino acids, hemoglobin, and horse serum proteins the values of  $\text{pK}_{\text{Am}}$  are all of the same magnitude, *i.e.* 5.3 to 6.3.

## THE PREPARATION AND SOME PROPERTIES OF LIVER FLAVIN

By F. J. STARE\*

(From the Laboratory of Biological Chemistry, Washington University  
School of Medicine, St. Louis)

By the following steps we obtain from liver of pig, beef, or horse rather more flavin with less effort than by methods previously

\* National Research Council Fellow.

described. The principal modification is the introduction of precipitation in alcoholic solution by  $\text{Ba}(\text{OH})_2$ , used by West and Howe in purification of the pernicious anemia factor. The efficiency of this step for flavin suggested a study of the possible relation of flavin to the pernicious anemia factor. Incubation of liver with pig stomach, said to increase the pernicious anemia factor, does not appreciably increase the flavin content. From results so far obtained with patients, liver flavin appears to have a very doubtful effect in pernicious anemia.

The steps used in our flavin preparation are extraction of ground liver (preferably horse liver) with boiling water, adsorption of the acidified filtrate with fullers' earth, elution by pyridine-alcohol-water (Salmon), precipitation of the eluate diluted with alcohol to 75 per cent by hot  $\text{Ba}(\text{OH})_2$ , solution of the precipitate in  $\text{H}_2\text{SO}_4$ , precipitation at pH 6 to 7 by  $\text{AgNO}_3$  (Kuhn), repeated extraction of Ag precipitate by hot 2 N acetic acid which dissolves most of the silver flavin, leaving the purines behind, removal of Ag by  $\text{H}_2\text{S}$ , and evaporation *in vacuo*. The residue is further purified. The flavins accompany purines during extraction and are separated completely from them with difficulty. Alcohol or acetone (70 to 80 per cent) and acetic acid extraction of the silver precipitate are serviceable.

#### FURTHER STUDIES ON MUSCULAR DYSTROPHIES, WITH REFERENCE TO INTOXICATION BY GUANIDINE AND SIMPLE GUANIDINE DERIVATIVES

By M. X. SULLIVAN

(From the Chemo-Medical Research Institute, Georgetown University,  
Washington)

Free guanidine treated with 1,2-naphthoquinone-4-sodium sulfonate and alkali gives a brown color on short heating or 15 minutes standing at room temperature. Then on addition of concentrated nitric acid, the guanidine solution becomes a good red, while all other compounds tested become light yellow with the exception of ammonia, indole, and to some degree methylamine. The urine in muscular dystrophies contains no free guanidine but does contain a simple guanidine derivative which on oxidation by  $\text{HgO}$  or  $\text{Ag}_2\text{O}$  gives free guanidine identified by the character of the picrate, the melting point, and the highly specific color reaction which is not given by complex guanidines. Under these conditions there

are no known interferers. From normal urine and from the urine in myasthenia gravis and from several cancer urines, methylguanidine was obtained. This does not give the characteristic picrate and does not give the guanidine color reaction. Under the treatment no guanidine could be obtained from creatine, creatinine, arginine, or any compound tested except glycocyamine. Feeding glycocholl over a relatively long period did not free the urine from simple guanidines. In one patient with progressive muscular dystrophy given glycocholl pills simple guanidines disappeared from the urine and some improvement resulted in general condition.

#### COMPARATIVE STUDY OF THE WOOL OF LAMBS ON ADEQUATE AND INADEQUATE RATIONS

By M. X. SULLIVAN, W. C. HESS, J. I. HARDY, AND PAUL E. HOWE

*(From the Chemo-Medical Research Institute, Georgetown University, and the Bureau of Animal Industry, United States Department of Agriculture, Washington)*

Cystine and total sulfur were determined on wool from lambs that received for 208 days (a) an adequate ration and (b) the same ration, but in amounts that did not permit of increase in the weight of the animals. More wool was obtained from the animals on the adequate ration. The whole fibers in both cases indicated approximately the same relative cystine content when analyzed by several cystine methods (Sullivan, Okuda, Folin-Marenzi, and by considering total sulfur as cystine). However, when the wool was divided into outer, middle, and body thirds, differences were found between the respective thirds. All methods show that the cystine content of the body third on the adequate diet is considerably and consistently higher than that of the body third on the inadequate ration. The ratios found for the body third for inadequate and adequate rations by the different methods are: Sullivan 100:110.5, Okuda 100:112.9, Folin-Marenzi 100:115.4, total sulfur 100:111.9.

Cross-sections of the wool fibers showed that the diameters of the wool of the underfed lambs were less than those of the full fed lambs, but there was no difference in the structure nor evidence of increased medullary space when the fibers were examined either in cross-section or longitudinally by polarized light. It appears, therefore, that the lowered cystine content is the result of the

change of the protein of the cortex and not of an increased medullary space.

### THE MEASUREMENT OF SERUM VOLUME

By F. WILLIAM SUNDERMAN

*(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia)*

In the determination of the serum volume by the intravenous injection of vital red, there is evidence to suggest that complete mixing of the dye in the circulating serum does not occur for 20 to 30 minutes after the injection. If samples of serum are obtained under basal conditions at intervals of 30, 60, and 90 minutes after the introduction of the dye, a linear relationship is obtained which permits extrapolation to the moment of injection and the estimation of the concentration of dye that would have been obtained were complete mixing instantaneous. The quantity of dye injected is measured with precision by means of an especially constructed burette. The colorimetric readings are made on undiluted serum in a colorimeter having two chambers on each side and fitted with a green monochromatic filter. A simplified procedure requiring only two samples of blood is proposed for clinical studies.

### BLOOD SUGAR AND RESPIRATORY EXCHANGE DURING HIGH CARBOHYDRATE INGESTION

By JOHN H. TALBOTT

*(From the Massachusetts General Hospital, Boston)*

Three male subjects undernourished but otherwise normal were studied over a period of 5 to 7 weeks, on a diet containing more than 600 gm. of carbohydrate daily, together with adequate amounts of protein, fat, and minerals. The fasting blood sugar concentration and the fasting respiratory exchange were determined semiweekly, the latter for a continuous 50 minute period. The gain in weight of the three subjects was 8.0, 6.8, and 12.4 kilos, respectively.

The first two subjects were given dextrose, and never showed any sugar in the urine. The third subject was given sucrose and showed a trace of urinary sugar on five occasions during the last



2 weeks of his study. At the beginning the respiratory quotient was below 0.85 in all subjects. It rose above 1.0 in the first days of the study, but to a different level in each subject. In the subjects given dextrose it reached a maximum of 1.05 and 1.04, respectively, in the 1st week and maintained this level for more than 2 weeks. This slowly fell to 0.96 and remained during the high dextrose ingestion. In the subject given sucrose it rose to 1.22 the 1st week and gradually fell to 0.90.

The blood sugar concentration was above 100 mg. per 100 cc. after the 1st week on the sucrose diet, but never exceeded 90 mg. on the dextrose diet; a return to low carbohydrate intake was followed by a diminished blood sugar concentration below 80 mg. A high carbohydrate diet may thus be given to non-diabetic individuals without producing untoward symptoms at any time.

#### THE EFFECT OF COMPLETE AND PARTIAL RENAL INSUFFICIENCY ON THE ACTION OF PARATHYROID HORMONE IN THE DOG

BY WILBUR R. TWEEDY, R. D. TEMPLETON, AND F. A. MCJUNKIN

*(From the Departments of Physiological Chemistry, Physiology, and Pathology, Loyola University School of Medicine, Chicago)*

The effects of parathyroid hormone, insulin, and adrenalin have been studied in a group of twenty-eight dogs in which complete or partial renal insufficiency had been produced surgically.

The action of single doses, or of massive multiple doses, of parathyroid hormone alone were followed in twelve dogs in the absence of kidney function by frequent determination of serum calcium and serum inorganic phosphate, and by histological examination of susceptible tissues. Two of these animals were made hypercalcemic by parathyroid hormone before bilateral nephrectomy, and afterward a simultaneous hypercalcemia and hyperphosphatemia persisted for more than 30 hours without apparent injury to heart or stomach tissues.

In four other dogs, bilaterally nephrectomized, calcium gluconate was injected, intramuscularly, and in two of them, parathyroid hormone was injected subcutaneously. In none of the experiments in which parathyroid hormone was administered after the suppression of kidney function was convincing evidence obtained that the hormone had mobilized calcium from the tissues.

Unilateral nephrectomy and partial impairment of the remaining kidney did not prevent hormone action.

Insulin and adrenalin, when administered to bilaterally nephrectomized dogs, were found to act in characteristic manner on the blood sugar, but the inorganic phosphate was irregularly affected, and the serum calcium was usually decreased.

#### SPECTROGRAPHIC STUDY OF THE CYTOCHROMES IN YEAST CELLS RESPIRING AT DIFFERENT TEMPERATURES

By FRANK URBAN

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis)

At 6°, 26°, and 34° previously washed and aerated bakers' yeast showed only the  $\alpha$  band of reduced cytochrome B at 565  $m\mu$  and a faint band between 535 and 545  $m\mu$ , a continuous stream of fine bubbles of  $O_2$  passing through the suspension. Addition of glucose to this suspension did not change the spectrum at 6°. However, at 26° the  $\alpha$  bands of reduced cytochromes B and C were present and, in addition, a narrow band at 537  $m\mu$ . At 34° the spectrogram showed the  $\alpha$  bands of reduced cytochromes A, B, and C in addition to faint bands at 537 and 523  $m\mu$ . The latter must be the  $\beta$  bands of reduced cytochromes B and A, while the broad band between 535 and 545  $m\mu$  represents the band of oxidized cytochrome C fused with the  $\beta$  band of reduced cytochrome B. Addition of KCN appears to shift the latter band several  $m\mu$  towards the short wave region. The  $O_2$  consumption of washed and aerated yeast was measured between 6–34°. A single straight line was obtained when the logarithm of  $O_2$  consumption was plotted against  $1/T$ . Stier found three lines of different slopes by plotting the logarithm of  $O_2$  consumption of yeast plus glucose against  $1/T$ . The results can be accounted for by assuming that in the presence of glucose the rate of respiration is controlled at the three temperatures by the rate of reduction of cytochrome C, cytochrome A, and *Atmungsferment*, respectively.

**THE RÔLE OF THE ACETYL DERIVATIVE AS AN INTERMEDIARY  
STAGE IN THE BIOLOGICAL SYNTHESIS OF AMINO  
ACIDS FROM KETO ACIDS**

BY VINCENT DU VIGNEAUD AND OLIVER J. IRISH

(From the Department of Biochemistry, School of Medicine,  
George Washington University, Washington)

Some years ago Knoop proposed the theory that the body synthesized amino acids from the corresponding keto acids by way of the acetyl amino acid. According to his theory the acetyl derivative of the amino acid might be formed by a condensation between the keto acid, ammonia, and pyruvic acid analogous to the *in vitro* synthesis of acetylalanine described by de Jong. Unfortunately Knoop abandoned the theory because of certain observations that were made in extending the studies on phenylaminobutyric acid to phenylalanine. However, we propose to show that the adverse conclusion was based on a misconception with regard to the spatial configuration of the acetylphenylalanine excreted after the feeding of acetyl-*dl*-phenylalanine, and that actually the data were in support of the theory. The purpose of the present paper is therefore an attempt to reestablish Knoop's theory.

Furthermore, it occurred to us that if the above theory of Knoop's were correct, the feeding of *l*-phenylaminobutyric acid should be followed by the excretion in the urine of the acetyl derivative of *d*-phenylaminobutyric acid. It was expected that the *l*-phenylaminobutyric acid would be deaminized by oxidation to the keto acid and then the acetyl derivative of the dextro isomer should be asymmetrically synthesized. This would serve as a crucial test of the above theory and, furthermore, offer a means of demonstrating the biological conversion of one isomer into the other. After feeding pure *l*-phenylaminobutyric acid, we were able to isolate from the urine the acetyl derivative of *d*-phenylaminobutyric acid.

**MINERAL METABOLISM OF TWENTY-THREE ADOLESCENT GIRLS,  
FIVE NEPHROTIC CHILDREN, AND TWO BOYS WITH PRO-  
GRESSIVE PSEUDOHYPERTROPHIC MUSCULAR DYSTROPHY**

By CHI CHE WANG, MILDRED KAUCHER, AND MARY WING

*(From the Children's Hospital Research Foundation and the Department of  
Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)*

This report concerns the metabolism of calcium, phosphorus, sodium, potassium, and magnesium.

*Adolescent Girls*—These results are correlated with weight, height, intake, gain in weight, and degree of underweight.

The average intakes in mg. per kilo of body weight per 24 hours are calcium 42, phosphorus 43, sodium 63, magnesium 8, and potassium 83. The corresponding values of urinary output are 3.4, 26, 59, 3.3, and 82. Those of fecal output are 27, 14, 0, 4.1, and 6. The values for retention are 11, 5, 3, 0.4, and -5.

*Nephrotic Children*—With the exception of sodium, the intake per kilo of all the minerals ran from 30 to 60 per cent higher in nephrotic children than in the girls. Their fecal mineral output ran markedly higher than that of adolescent girls. Urinary calcium was unusually low, 0.7 mg. per kilo, and fecal sodium high, 153 mg. per 24 hours. The retention of minerals in nephrosis differs little from that in normal children; calcium is slightly lower and magnesium higher.

*Progressive Pseudohypertrophic Muscular Dystrophy*—The intakes corresponded to those for the nephrotic group. With the exception of sodium, the mineral metabolism of the two boys varied little. Urinary calcium was higher, 8.1 mg. per kilo, and the retention of both calcium and phosphorus lower than that of either the adolescent girls or the nephrotic children, calcium being 4 mg. and phosphorus 1 mg.

**THE EFFECT OF ADMINISTRATION OF PREPARATIONS OF  
GROWTH HORMONE OF THE ANTERIOR LOBE OF THE  
PITUITARY UPON GESTATION AND THE WEIGHT OF  
THE NEW-BORN (ALBINO RATS)**

By RUTH M. WATTS

*(From the Department of Obstetrics and Gynecology of the University of  
Chicago, Chicago)*

The study of the interrelationship of the pituitary and pregnancy has led to investigations of the effect of the growth hormone

of the anterior lobe upon gestation and the development of the young. The present study, supplementing the work of Teel with crude extract, was undertaken at the suggestion of Dr. Fred L. Adair.

Growth hormone preparations made and assayed according to the method of van Dyke and Wallen-Lawrence were administered subcutaneously daily for varying periods to pregnant rats with histories of normal reproduction and litters. The administration of some preparations resulted in prolonged gestation, protracted parturition, and still-born young, as reported by Teel, Hain, and Sontag and Munson; the use of other preparations permitted normal parturition at term.

Increases in maternal weight and in the weight of the new-born of the latter group have been given statistical consideration. Significant increases in the maternal gain during gestation and in the "permanent" gain were observed. The mean weight of the young showed an increase of 10.9 per cent over that of the control group. Comparison of these means by the *t*-test method (Fisher) gives a probability of  $<0.000$  that these values could have resulted from random sampling.

The compiled statistics of control groups showed (1) that both the maternal weight changes and the weight of the new-born may vary with the number of the pregnancy, and (2) that the mean weight of the new-born per litter is a more characteristic value than the litter weight.

#### THE PRESENCE OF GLYCOCYAMINE IN URINE

By C. J. WEBER

*(From the Department of Internal Medicine, University of Kansas School of Medicine, Kansas City)*

The isolation of glycocyamine (guanidoacetic acid) from the urine of two cases of pseudohypertrophic muscular dystrophy has been described.<sup>28</sup> A continuation of this problem has resulted in the isolation of this compound from normal human urine and also from the urine of two dogs which was collected during a period of fasting. My results indicate that glycocyamine is a regular constituent of normal urine and that its presence is not a result of its ingestion preformed in the diet.

<sup>28</sup> Weber, C. J., *Proc. Soc. Exp. Biol. and Med.*, **32**, 172 (1934).

A method for the colorimetric determination of glycoeyamine in urine has been developed which is based on the adsorption of this compound with Lloyd's reagent and its subsequent elution. The color is developed in the final filtrate from Lloyd's reagent by the application of a modified Sakaguchi reaction. This method indicates that 20 to 40 mg. of glycoeyamine are excreted daily by a normal adult. In one case of pseudohypertrophic muscular dystrophy, an increase of 75 per cent in the glycoeyamine excretion was indicated by this method following the administration of glycine.

Glycoeyamine formation in the body probably represents a phase in the intermediary metabolism of which creatine is the end-product. The excretion of glycoeyamine in the urine would therefore represent an overflow phenomenon of an intermediate metabolic product produced slightly in excess of the needs of the body.

#### PRECIPITATING AGENTS FOR USE IN THE ESTIMATION OF SUGARS IN BIOLOGICAL MATERIALS

BY EDWARD S. WEST, RUTH A. LANE, AND GEORGE H. CURTIS

*(From the Biochemical Laboratories, Washington University School of Medicine, St. Louis, and the University of Oregon Medical School, Portland)*

The efficiencies of a number of precipitation procedures designed to yield filtrates satisfactory for sugar analysis and containing little or no added electrolytes have been investigated. The work is a continuation of the study begun by the senior author a number of years ago.

The precipitating agents compared were: (1) a saturated solution of  $\text{HgSO}_4$  in 10 per cent  $\text{H}_2\text{SO}_4$  (West, Scharles, and Peterson); (2) a saturated solution of  $\text{HgSO}_4$  in a 21 per cent  $\text{Fe}_2(\text{SO}_4)_3$  solution in 1 N  $\text{H}_2\text{SO}_4$  (about 25 per cent  $\text{HgSO}_4$ ); (3) a 21 per cent solution of  $\text{Fe}_2(\text{SO}_4)_3$ ; (4) Reagent 2 plus Lloyd's reagent; (5) Reagent 3 plus Lloyd's reagent.

Each precipitating solution was added to the fluid (urine, hydrolyzed tissue, mixtures of urea, creatinine, creatine, etc.) and then neutralized with  $\text{BaCO}_3$ . When used, the Lloyd's reagent was added before the  $\text{BaCO}_3$ , the mixture shaken, allowed to stand a few minutes, and then neutralized with  $\text{BaCO}_3$ . Mercury was removed with  $\text{H}_2\text{S}$ .

Reagents 2 and 4 were found most efficient in removing urea, creatine, and creatinine, the latter reagent being slightly better for

the removal of creatinine. Both reagents are far superior to Reagent 1.

Reagent 4 was slightly more efficient in removing non-fermentable reducing substances from urine and hydrolyzed muscle and liver than Reagent 2. They were both decidedly better than Reagent 1.

Reagent 1 was only slightly superior to Reagent 5 in removing non-fermentable reducing substances from urine and hydrolyzed tissues.

### RELATION OF VITAMIN B ( $B_1$ ) TO FAT METABOLISM

#### I. THE RÔLE OF FAT IN THE REFLECTION PHENOMENON

BY DOROTHY V. WHIPPLE AND CHARLES F. CHURCH

*(From the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia)*

The feces of rats on a vitamin B ( $B_1$ )-deficient diet, containing 10 per cent lard with sucrose as the only source of carbohydrate, contained the thermolabile factor which alleviated the symptoms of beriberi. When the diet was made fat-free, this anti-beriberi factor was not present in the feces. When the sugar in the fat-free diet was replaced by corn-starch, a small amount of this fecal factor was present. Corn-starch contains a small amount of fat within the starch globule, which is not removed by extraction with fat solvents, but which becomes available to the animal during digestion when the globule is ruptured.

The symptoms of vitamin B ( $B_1$ )-deficiency were produced more quickly on fat-free diets than on diets containing fat. The larger the amount of fat the longer was the time interval before symptoms became manifest, as has been demonstrated previously. Fat alleviated the symptoms of beriberi in rats permitted to develop the disease on a fat-free diet, although they eventually succumbed to the avitaminosis.

The amount of anti-beriberi factor eliminated in the feces of vitamin B-deficient rats is directly correlated with the amount of lard in the diet. This factor is not fat itself, since the ether-extracted dried feces were more potent in alleviating the symptoms of beriberi than was the ether extract.

From these experiments it is concluded that fat is essential for

the production of the anti-beriberi factor in the gastrointestinal tract of the vitamin B-deficient rat.

## THE CHEMISTRY OF HUMAN EPIDERMIS

### II. THE ISOELECTRIC POINT OF THE STRATUM CORNEUM AND OTHER HUMAN KERATINS AS DETERMINED BY ELECTROPHORESIS

By VERNON A. WILKERSON

*(From the Department of Physiological Chemistry, Howard University Medical School, Washington)*

The finely divided stratum corneum from normal human epidermis and from patients having dermatitis exfoliativa was suspended in buffer solutions of known pH and the electrophoretic velocities determined. The curves of mobility (in  $\mu$  per second per volt per cm.) plotted against pH were constant for all the samples examined regardless of the source. The value assigned to the isoelectric point was pH 3.8.

The isoelectric points of human hair and finger nails, both of which are specialized and highly keratinized appendages of the epidermis, compared quite favorably with that of the stratum corneum. This group of keratins has been shown to contain histidine, lysine, and arginine in the same molecular ratios, while the relative amounts of other amino acids present are variable. It is suggested that the charges on the particle surfaces of these embryologically related keratins and consequently their isoelectric points are determined by the basic amino acid "anlage" characteristic of the group, and only slightly or not at all affected by differences in other amino acids.

### THE ACTION OF MALE AND FEMALE HORMONES UPON THE REPRODUCTIVE GLANDS AND DUCTS OF THE CHICK EMBRYO

By B. H. WILLIER, T. F. GALLAGHER, AND F. C. KOCH

*(From the Department of Zoology, The University of Rochester, Rochester, New York, and the Department of Physiological Chemistry and Pharmacology, the University of Chicago, Chicago)*

A single injection of 0.1 cc. of sex hormone solution (aqueous or ethylene glycol) of varying potency was made into the albumin of



eggs of 24 hours incubation and development continued until the 19th day (*cf.* Kozelka and Gallagher, 1934). The eggs used came from brown Leghorns and from a sex-linked cross. Over 350 embryos have been examined.

Theelin and theelol (0.05 to 2.0 mg. per egg) do not affect the size, form, or histology of the left and right ovaries of genetic females or the right testes of genetic males. The left testis, however, becomes changed in form to a flattened ovary-like structure consisting of both cortical and testicular tissues (ovotestis). In extreme cases (2.0 mg.) the testicular cords have been replaced largely by medullary cords. The difference in response of the two testes is attributed to the presence of a germinal epithelium (incipient cortex) on the left testis only during early development. In both sexes the Wolffian ducts appear unaffected. The oviducts persist in the males; with larger dosages they become enormously swollen in both sexes.

Male hormone from bull testis extracts (10 to 200 bird units) produced no observable effect on the gonads, or oviducts of either sex; Wolffian ducts may be modified. The same concentrations of male hormone from human urine extracts produced no changes in the female gonads but caused the formation of cortex on the left male gonad and a flattened form, whether or not female hormone is present. The Wolffian ducts in both sexes are consistently and tremendously swollen. The oviducts of the females seem inhibited; *i.e.*, rudimentary or absent altogether.

#### CHEMICAL INVESTIGATIONS ON THE CORTICAL HORMONE OF THE ADRENAL GLAND

By OSKAR WINTERSTEINER AND J. J. PFIFFNER

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Concentrates assaying 100 to 200 dog units per mg. have been prepared from large quantities of permutit-purified material by distribution procedures previously described. Further fractionation has demonstrated that neither the factor yielding precipitates with  $\alpha$ -naphthol nor the factor which reduces silver solution at pH 8 is concerned with the activity. Potent products have been





